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PRINCIPAL INVESTIGATOR: Samuel R. Denmeade, M.D.

CONTRACTING ORGANIZATION: Johns Hopkins University
Baltimore, Maryland 21205

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6. AUTHOR(S)
Samuel Denmeade, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Johns Hopkins University
Baltimore, Maryland 21205

E-Mail: denmesa@jhmi.edu

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13. ABSTRACT (Maximum 200 Words)
The underlying hypothesis of this proposal is that a breast tissue/cancer proteolytic activity can be identified by screening the extracellular fluid from human breast cancers with a fluorescence quenched random peptide library. The peptide substrate(s) identified from this screening could be used to produce prodrugs that are targeted for specific activation by proteolytic activity present in extracellular fluid of breast cancers while avoiding systemic toxicity. In the first year we developed methods to synthesize large fluorescently quenched peptide libraries as outlined in task 1. In preliminary studies, we incubated these libraries with a purified serine protease, human glandular kallikrein 2, and identified a series of putative substrates. These substrates were resynthesized as soluble peptides and peptides were then identified that were excellent hK2 substrates but unstable to non-specific hydrolysis in human plasma. Coupling these peptides to the thapsigargin analog, L12ADT, however, produced a prodrug that was readily hydrolyzed by hK2, stable in human plasma in vitro and mouse plasma both in vitro and in vivo, and was selectively cytotoxic to cancer cells in the presence of enzymatically active hK2. These studies demonstrated the feasibility of the approach to identification of protease substrates outlined in tasks 1-3 of the proposal. However, incubation of breast cancer homogenates or concentrated media from breast cancer cell lines did not yield any hydrolyzed peptides. This lack of hydrolys is most likely is due to a combination of ng/ml concentrations of protease in the extracellular fluid and the need to dilute samples to cover entire bead library (i.e. 10-40 mls). A new approach for combinatorial screening is required that would yield libraries of small physical volume to screen small volume of extracellular fluid from breast cancers without requiring substantial dilution. To accomplish this, we will use the recently described combinatorial technique of in vitro compartmentalization that allows for rapid screening of large libraries in volumes of < 100 µl.

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INTRODUCTION:

From a very early stage in the disease process, breast cancers are composed of a heterogeneous collection of cells. These breast cancer cells within a given patient have varying sensitivities to commonly used antiproliferative agents. Metastatic breast cancers are often initially responsive to the commonly used chemotherapeutic agents such as doxorubicin and the taxanes. Unfortunately, no patient is cured by these therapies and thus, metastatic breast cancer is uniformly fatal. New effective therapies for breast cancer are therefore urgently needed.

Currently used chemotherapies are for the most part antiproliferative agents and general cytotoxins that take advantage of the differential rate of growth of cancerous versus normal tissue. These therapies, therefore, are not breast cancer cell specific in their cytotoxicity and their use is often associated with significant dose limiting toxicities. New strategies are needed to inhibit breast cancer specific targets while at the same time avoiding toxicity to normal host tissues. The approach outlined in this proposal is to inhibit a ubiquitous intracellular protein whose function is mandatory for survival of all cell types. This approach would overcome the problem of heterogeneity and "resistance" as all cancer cells within a tumor could be killed via this approach. The obvious shortcoming of this approach is that the cytotoxicity would not be cell-type specific and administration of such a general toxin would be associated with significant systemic toxicity.

Previously we and others have identified such a ubiquitous intracellular protein, the Sarcoplasmic/ Endoplasmic Reticulum Ca^{2+} ATPase (SERCA) pump (1-4). We documented that inhibition of this SERCA pump by the natural plant product thapsigargin (TG) could induce apoptosis of all cell types including breast and prostate cancer cell lines (3-5). Because TG does not possess the primary amine needed for coupling to the C-terminal carboxyl of a peptide carrier, primary amine analogs of TG were made (6,7). Based on a model of the TG binding site within the SERCA pump it was determined that modifications of the TG molecule could possibly be made in the side chain in the 8-position without adversely effecting SERCA pump inhibitory activity (6,7). Using this rationale a series of TG analogs (i.e. ~50) modified in the 8-position with primary amine containing side chains were synthesized by my long time collaborator and discoverer of TG, Dr. Soeren Christensen from the Royal Danish School of Pharmacy in Copenhagen (6,7). These analogs were characterized for their ability to inhibit the SERCA pump and elevate intracellular calcium (6,7). In addition, these analogs were assayed for cytotoxic activity against androgen independent human prostate cancer cells in vitro (6,7). The best of these analogs contained a 12-amino dodecanoate side chain (12ADT) coupled to the amino acid leucine (L12ADT) and was found to have an LD_{50} value against prostate cancer cells of ~ 30 nM. This LD_{50} is identical to that reported for TG (25). In addition, we developed a strategy to target this potent TG analog specifically to sites of prostate cancer to avoid systemic toxicity (8). This approach targets the proteolytic activity of the serine protease prostate-specific antigen (PSA) (8). We identified a PSA specific peptide substrate (9) that is can be coupled to the L12ADT analog of TG to produce an inactive prodrug that can only be proteolytically activated by enzymatically active PSA present within the extracellular fluid prostate cancer (9-11).

Breast cancer cells, like prostate cancer, secrete a variety of proteases. While a PSA-like protease that is secreted in large amounts in a tissue restricted manner has yet to be identified for breast tissue, there are a number of proteases whose expression has been demonstrated to be relatively increased in breast cancer tissue. Examples of such proteases include cathepsins, kallikreins and members of the matrix metalloproteinase family (12-22). Although newer techniques like differential display and SAGE analysis are powerful tools that are yielding large numbers of putative new therapeutic targets, these techniques do not always provide information about functional activity of identified expression products. This functional information is particularly critical when evaluating protease expression because the activity of these proteins is tightly regulated at a number of levels (e.g. expression levels, processing to active protease, binding to inhibitors, auto-degradation). Therefore, additional methods are needed to that will help define not only the presence but also the functional activity of these proteases. The significance of this proposal is that it proposes a strategy to identify specific peptide substrates for breast cancer/tissue specific proteolytic activities. These studies may help to identify new diagnostic and/or therapeutic targets in breast cancer. In addition, the specific peptide substrates will be

incorporated into prodrugs to yield novel targeted therapies for breast cancer while avoiding toxicity to normal tissue.

BODY:

Hypothesis: The underlying hypothesis of this proposal is that a breast tissue/cancer proteolytic activity can be identified by screening the extracellular fluid from human breast cancers with a fluorescence quenched random peptide library. The peptide substrate(s) identified from this screening could be used to produce prodrugs that are targeted for specific activation by proteolytic activity present in extracellular fluid of breast cancers while avoiding systemic toxicity. The peptide substrate(s) will also be used to isolate and characterize specific protease responsible for specific substrate hydrolysis.

To accomplish the goal of the proposal, we outlined 4 tasks that would be completed over the three year funding period. The objective of **Task 1** (months 0-12) was to synthesize fluorescence quenched random peptide libraries to identify proteolytic activities present in the extracellular fluid of human breast cancer cell suspensions. The objective of **Task 2** (months 3-12) is to synthesize soluble fluorescent peptide substrates to characterize specificity and efficiency of hydrolysis. The objective of **Task 3** (months 9-24) is to synthesize thapsigargin prodrugs by coupling thapsigargin analog to lead peptide substrates identified in tasks 1-3. The objective of Task 4 (months 24-36) is to identify specific protease(s) responsible for proteolytic activity using peptide substrates identified in tasks 1-2.

As an initial step toward accomplishing we needed to develop expertise in the synthesis and characterization of large fluorescence quenched random peptide libraries of ~ 1.5- 2 million peptide sequences containing 6 random amino acids. To produce these libraries we used the "one bead-one peptide" splitting and mixing technique (23-24). This approach produces peptides bound to PEGA grafted "beads" in such a way that each individual bead contains many copies of one unique peptide sequence (25). The peptides are synthesized using a previously described approach with a fluorescent molecule [aminobenzoic acid (ABZ)] at the carboxy terminus and a quencher molecule (nitrotyrosine) at the amino terminus (26-29). This design produces a fluorescence quenched library because the emission spectrum of ABZ overlaps exactly with the absorbance spectrum of nitrotyrosine. Cleavage of the peptide sequence by a protease liberates the quencher moiety resulting in a fluorescent bead that is easily visible and easily removed for peptide sequencing, figure 1.

To accomplish task 1 we proposed to incubate these libraries with extracellular fluid from breast cancers. However, prior to exposing fluid from breast cancers that most likely would contain a mixture of proteases, we decided to work out the methodologies for synthesizing and screening a large random library using a more simplified system. Therefore, prior to screening the breast cancer fluid we chose to use the method to identify a substrate for a single purified protease. For these preliminary studies we selected the serine protease human glandular kallikrein 2 (hK2). HK2 has been well-characterized in prostate tissue but is also known to be selectively produced by ~ 50% of breast cancers. Through a collaboration with Dr. Hans Lilja at Memorial Sloan Kettering, we have access to large amounts of purified enzymatically active hK2. Therefore, in preliminary studies we generated a fluorescence quenched, combinatorial peptide library and incubated it with hK2. Following incubation, a series of putative peptide substrates were identified and resynthesized as soluble peptide substrates. These soluble substrates were screened for hK2 activity and plasma stability. Finally, the best of these substrates with the sequence Gly-Lys-Ala-Phe-Arg-Arg (GKAFRR) was coupled to a potent analog of thapsigargin to generate the prodrug GKAFRR-L12ADT. This prodrug was then characterized for hydrolysis by hK2, stability in human plasma and selective cytotoxicity against hK2 producing and non-producing cancer cell lines. The rationale, methods and results of these studies using hK2 are described in detail in the attached appendix (appendix 1) which is a manuscript that has been recently submitted for publication.

These studies using purified hK2 allowed our laboratory to develop expertise and techniques required to accomplish goals outlined in tasks 1-3 of the proposal. The results generated using the combinatorial library approach to identify hK2 peptide substrates suggested that a similar approach, as outlined in the proposal, could

successfully yield breast cancer specific substrates. Therefore, a large combinatorial library was synthesized containing ~ 1.5 million random sequences of 6 amino acids in length. Our original intention was to incubate this library with extracellular fluid obtained from fresh breast cancer specimens obtained directly from the operating room. Unfortunately, over the course of this first funding year, we have been unable to obtain such fresh samples from the Johns Hopkins Department of Pathology as anticipated. Instead, we have had to rely on frozen specimens to complete these screening assays. On this basis, the newly synthesized combinatorial library was initially incubated with homogenate from 2 breast cancer specimens (total of ~ 200 mg of cancer tissue). This homogenate was incubated with 25% of the peptide library, initially for a period of 48 hrs. At this point in time, no beads had become fluorescent, indicating that no hydrolysis had occurred. The library was incubated for a total of 5 days, again with no positive fluorescent beads.

One possible reason for lack of hydrolysis of peptide containing beads in this assay may be due to small amount of frozen cancer tissue that was available for the assay which, concomitantly, would contain low amounts of proteases. For this assay we obtained homogenized tissue in 1 ml of buffer. The peptide library of ~1 million beads requires ~ 40 ml of solution to wet all of the beads. Thus, the breast cancer homogenate had to be diluted ~ 10-fold more just to cover the beads in 25% of the library. Thus, low levels of protease present in the extracellular fluid would be diluted to levels that may lie below sensitivity of assay, even after 5 days incubation. For example, hK2 is present in media of prostate cancer cells at levels of ~ 10-50 ng/ml. Dilution of this media ~ 10-fold would yield a concentration of hK2 of ~ 1- 5 ng/ml. In the combinatorial library screen a level of 4 μg/ml hK2 was required to identify hydrolyzed peptides over 48 hr incubation period. In the original proposal our plan was to use media from single cell suspension of human breast cancers that had been conditioned for multiple days as the source of extracellular protease. Due to the difficulty in obtaining such fresh specimens we opted to use a human breast cancer cell line, MCF-7, to determine if this combinatorial peptide methodology could be used to identify proteolytic activity. For these assays, MCF-7 cells were grown in standard serum containing media until cells were ~60% confluent. Cells were then transferred into DMEM media without any additional serum to obtain conditioned media that did not have any serum protease inhibitors present. Media was conditioned for 4 days and then media from 5 T-75 flasks (i.e. ~ 5 x 10^7 cells) was collected and concentrated ~8-fold to a volume of 10 ml. Again, this fluid was incubated with beads representing ~ 25% of the library (i.e. ~ 250,000 peptide sequences). After 5 days incubation, 3 fluorescent beads were observed, but Edman sequencing revealed that these beads represented false positives that had not been correctly synthesized.

On the basis of the preliminary studies with hK2 and these two experiments with frozen breast cancer tissue and concentrated conditioned media from a human breast cancer cell line, we have concluded that the combinatorial bead strategy for identifying breast cancer proteases has a number of limitations and will need to be modified in future studies. The major limitations of this approach are:

1. The beads used for solid phase synthesis are ~ 300 micrometers in diameter and therefore, one million beads takes up ~ 40 ml volume. This requires either a large amount of extracellular fluid (ECF) or dilution of ECF multiple-fold to cover all of the beads.

2. The proteases in the ECF of breast cancer cells most likely are in concentrations in the ng/ml range and this bead methodology appears to work best with proteases in the μg/ml range.

3. Proteases greater than ~ 35,000 MW do not easily access the central portions of the beads, resulting in limited hydrolysis and poor sensitivity.

4. Steric constraints imposed by the solid phase surface limit peptide hydrolysis compared to soluble peptide substrates.

Therefore, future studies will require development of methods to either decrease the physical size of the library or to screen soluble peptides in smaller volume of fluid that can be obtained from ECF of human breast

cancers. To accomplish this tasks we intend to modify the combinatorial strategy originally outlined in task 1 and adopt the combinatorial screening method recently described by Sepp, et al. (see appendix 2) depicted in Figure 1 (30). In this approach, an oligonucleotide is generated that encodes a random peptide flanked by peptide tags recognized by commercially available antibodies. This oligonucleotide is coupled to biotin and then attached to a streptavidin coated microbead (i.e. one micron diameter). Beads are then compartmentalized in a water-oil emulsion to give on average ~ 1 bead per compartment and are transcribed and translated in the compartment. For our application the gene will encode for a peptide with the general sequence FLAG Tag- Random 8 amino acid Peptide- HA Tag. Peptides will be bound to bead by binding to commercially available biotinylated antibody to FLAG attached to streptavidin surface. Beads are then incubated with protease containing ECF from breast cancers. Non-hydrolyzed beads will bind to commercially available HRP-linked HA antibody and will become fluorescent. Beads containing peptides that have been hydrolyzed by proteases will not bind to HRP-linked HA antibody and will not be fluorescent. These beads can then be rapidly sorted on flow cytometer. Non-fluorescent beads can be captured and nucleotide sequence determined to then determine random peptide sequence. These peptides can then be resynthesized as soluble peptides and rescreened as outlined in task 2. Further modifications to peptide sequence can be made suing this approach to identify an optimal peptide for protease activity present in ECF.

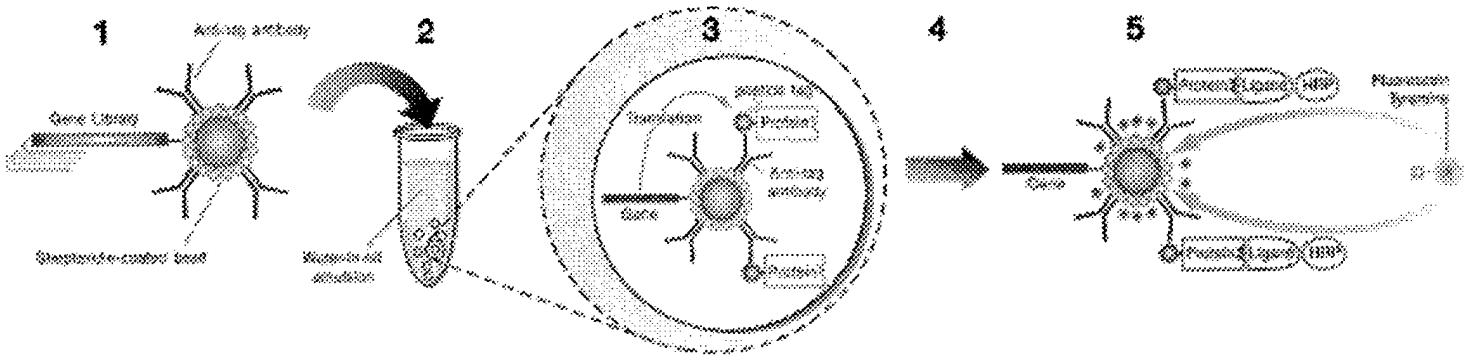

Figure 1. Creation of microbead display libraries by In Vitro Compartmentalisation and selection for binding using flow cytometry. A repertoire of genes encoding protein variants, each with a common N- or C-terminal epitope tag, are linked to streptavidin-coated beads carrying antibodies that bind the epitope tag at, on average, ≤1 gene per bead (1). The beads are compartmentalised in a water-in-oil emulsion to give, on average, <1 bead per compartment (2), and transcribed and translated in vitro in the compartments. Consequently, in each compartment, multiple copies of the translated protein become attached to the gene that encodes it via the bead (3). The emulsion is broken (4), and the microbeads carrying the display library isolated. The beads are incubated with ligand coupled to horseradish peroxidase (HRP), washed to remove unbound ligand and incubated with hydrogen peroxide and fluorescein tyramide (5). Immobilised HRP converts the fluorescein tyramide into a short-lived, free-radical intermediate which reacts with adjacent proteins. Hence, beads displaying proteins that bind ligand become labeled with multiple fluorescein molecules. These beads can then be enriched (together with the genes attached to them) by flow cytometry. [Figure and legend from Sepp et al. FEBS Letters 532:455-458, 2002 (ref 30 and Appendix 2)].

There are many advantages to the In Vitro Compartmentalisation approach for this application. First, the physical volume of the library is greatly reduced by decreasing the size of the bead particles from 300 to 1 micron in diameter. A library of ~ one million sequences will only take up ~50-100 µl volume in this approach (31). In addition, the use of an oligonucleotide to generate the peptide will allow for rapid modification and optimization of peptide sequences. The time to determine peptide sequence and costs of sequencing will be greatly reduced by this approach. Finally, bead screening and sorting can be performed using standard flow cytometer available as a

core facility within the Oncology Department. This method, therefore, allows for rapid screening of large numbers of beads in a short period of time.

KEY RESEARCH ACCOMPLISHMENTS:

1. Synthesized and characterized a large fluorescently quenched combinatorial peptide library and successfully identified a substrate for the serine protease human glandular kallikrein 2 (hK2), which is expressed by human prostate cancers and by ~ 50% of human breast cancers.
2. Characterized hK2 hydrolysis of soluble hK2 substrates and determined plasma stability
3. Synthesized a hK2-activated thapsigargin prodrug that is efficiently hydrolyzed by hK2 and stable in human plasma
4. Demonstrated that this hK2 prodrug is selectively toxic in the presence of enzymatically active hK2.
5. Screened homogenates of frozen human breast tumors and conditioned media from the human breast cancer cell line MCF-7 but did not identify a putative peptide substrate for a breast cancer protease.

REPORTABLE OUTCOMES:

Presentations:

Janssen S, **Denmeade SR.** Identification of Tumor Associated Protease Substrates Using Combinatorial Chemistry. AACR/EORTC Molecular Targets and Cancer Therapeutics Meeting, Frankfurt, Germany 2002.

Manuscripts and Abstracts:

1. Janssen S, **Denmeade SR.** Identification of Tumor Associated Protease Substrates Using Combinatorial Chemistry. Eur J Cancer 38 Suppl 7:S97, 2002.
2. **Denmeade, S.R.,** Sokoll, L.J., Dalrymple, S., Rosen, D.M., Gady, A.M., Bruzek, D., Ricklis, R.M., Isaacs, J.T. Dissociation Between Androgen Responsiveness for Malignant Growth vs. Expression of Prostate Specific Differentiation Markers PSA, hK2 and PSMA in Human Prostate Cancer Models. Prostate, 2003;54:249-257.
3. **Denmeade, S.R.,** Jakobsen, C., Janssen, S., Khan, S.R., Lilja, H., Christensen, S.B. and Isaacs, J.T. Prostate-Specific Antigen (PSA) Activated Thapsigargin Prodrug as Targeted Therapy for Prostate Cancer, J Natl Cancer Inst 2003;95:990-1000.
4. Janssen S, Jakobsen CM, Rosen DM, Reineke U, Christensen SB, Lilja H, **Denmeade SR**. Screening a combinatorial peptide library to develop a human glandular kallikrein-2 activated prodrug as targeted therapy for prostate cancer, Submitted for publication, 2004.

Employment:

Support for Post-Doctoral Fellow, Dr. Samuel Janssen to carry out experiments outlined in this proposal from May 1, 2003 to August 30, 2003. Dr. Janssen subsequently obtained a senior staff scientist position at Amylin, Inc., San Diego, CA.

Salary support for graduate student, Aaron LeBeau, beginning 1/1/04 to complete tasks 1-3 outlined in proposal.

CONCLUSIONS:

At the end of one year of funding we have developed methods to synthesize large fluorescently quenched peptide libraries as outlined in task 1. In preliminary studies, we incubated these libraries with a purified serine protease, human glandular kallikrein 2, and identified a series of putative substrates. These substrates were resynthesized as soluble peptides and characterized for hK2 hydrolysis and plasma stability. Peptides were then identified that were excellent hK2 substrates but unstable to non-specific hydrolysis in human plasma. Coupling these peptides to the thapsigargin analog, L12ADT, however, produced a prodrug that was readily hydrolyzed by hK2, stable in human plasma in vitro and mouse plasma both in vitro and in vivo, and was selectively cytotoxic to cancer cells in the presence of enzymatically active hK2. These studies demonstrated the feasibility of the approach to identification of protease substrates outlined in tasks 1-3 of the proposal. However, incubation of breast cancer homogenates or concentrated media from breast cancer cell lines did not yield any hydrolyzed peptides. This lack of hydrolysis most likely is due to combination of ng/ml concentrations of protease in extracellular fluid and need to dilute samples to cover entire bead library (i.e. 10-40 mls). A new approach for combinatorial screening is required that would yield libraries of small physical volume to screen small volume of extracellular fluid from breast cancers without requiring substantial dilution. To accomplish this, we will use the recently described combinatorial technique of in vitro compartmentalization that allows for rapid screening of large libraries in volumes of < 100 µl.

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Screening a combinatorial peptide library to develop a human glandular kallikrein-2 activated prodrug as targeted therapy for prostate cancer

Samuel Janssen, Carsten M. Jakobsen, D. Marc Rosen, Ulrich Reineke, Soeren B. Christensen, Hans Lilja, and Samuel R. Denmeade
The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, MD (SJ, DMR, SRD); The Danish University of Pharmaceutical Sciences, Copenhagen, Denmark (CMJ, SBC); Jerini Biotools, Berlin, Germany (UR); Memorial Sloan Kettering Hospital, New York NY (HL)

Abstract
Prostate cancer cells secrete unique proteases that represent targets for the treatment of prostate cancer. In this study, one of these proteases, human glandular kallikrein 2 (hK2), was investigated as a novel proteolytic activator of prodrug therapeutic agents. To identify peptide sequence requirements for hK2, we used a combination of membrane bound peptides (SPOT analysis) and combinatorial chemistry utilizing fluorescence-quenched peptide substrates. Both techniques indicated that a peptide with two arginines N-terminal of the scissile bond produced the highest rates of hydrolysis. A lead peptide substrate with the sequence Gly-Lys-Ala-Phe-Arg-Arg (GKAFRR) was hydrolyzed by hK2 with a Km of 26.5 mM, kcat of 1.09 sec-1 and a kcat/Km ratio of 41,132 sec-1 M-1. The GKAFRR peptide was coupled to L12ADT, a cytotoxic analog of the natural plant product thapsigargin. Thapsigargin is a potent inhibitor of the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) pump that induces cell proliferation independent apoptosis through dysregulation of intracellular calcium levels. Kinetic analysis showed that the prodrug was rapidly hydrolyzed by hK2. Plasma stability studies indicated that the prodrug was stable in plasma for at least 24 hours, whereas the GKAFRR-L peptide substrate was unstable in human plasma. The hK2-activated thapsigargin prodrug was not activated by cathepsin B, D and urokinase but was an excellent substrate for plasmin. The GKAFRR-L12ADT was selectively cytotoxic in vitro to cancer cells in the presence of enzymatically active hK2.

Introduction

Non-organ confined prostate cancer is uniformly fatal once it reaches the hormone-refractory state because current therapies are unable to completely eliminate the androgen independent prostate cancer cells present within metastatic sites. In previous studies, our laboratory and others have demonstrated that metastatic androgen independent prostate cancer cells have a remarkable low rate of cell proliferation (1, 2) . This low proliferative rate could explain the relative unresponsiveness of these cells to standard anti-proliferative chemotherapy, while highly proliferative androgen independent prostate cancer cell lines remain exquisitely sensitive to apoptosis induction in vitro.

In contrast to agents that activate apoptosis in proliferating cells, our laboratory has shown that thapsigargin (TG), a potent inhibitor of the sarcoplasmic/endoplasmic reticulum ATP-dependent Ca^{2+} (SERCA) pumps (3-5), has the dose-response ability to elevate intracellular calcium (Ca_i) to sufficient levels to induce apoptosis in all of the rodent and human androgen independent prostate cancer cell lines without requiring the cells to be proliferating (3, 6, 7). The cytotoxicity of TG, however, is not prostate cancer cell type specific (8, 9) . Therefore, TG would be difficult to administer systemically without significant side effects. In addition, TG is sparingly water soluble due to its high lipophilicity. Therefore, a method is required that both better solubilizes TG as well as selectively targets TG's cytotoxicity to metastatic deposits of androgen independent prostate cancer cells systemically (10). To accomplish this, a primary amine containing TG analog can be coupled to a peptide carrier to produce water soluble inactive prodrug that is selectively activated within sites of prostate cancer (8, 10). The peptide carrier in this approach is designed to be a selective substrate for prostate tissue specific proteases such as prostate-specific antigen (PSA) or human glandular kallikrein 2 (hK2) (10). PSA and hK2 are only produced in high levels by normal and malignant prostate cancer cells (11-14). In addition, metastatic prostate cancer cells continue to secrete enzymatically active PSA and hK2 into the extracellular fluid at high levels (11, 12, 15). Once in the extracellular fluid, enzymatically active PSA and hK2 eventually enter the blood where they are inactivated by binding to major serum protease inhibitors [i.e.α1-antichymotrypsin and α2-macroglobulin for PSA (14, 16-19) and α1- antichymotrypsin, α2-antiplasmin, antithrombin II, protein C inhibitor, and α2-macrogloblin for hK2 (20, 21).

Ideally, the TG prodrug is inactive until the TG analog is liberated in the presence of enzymatically active PSA or hK2. In previous studies, we synthesized and characterized a series of primary amine containing TG analogs and identified one that is a highly potent inhibitor of the SERCA pump and as equally cytotoxic as TG (22, 23). In additional studies, we identified a six amino acid peptide substrate that is efficiently hydrolyzed by PSA (24). The potent TG analog and other cytotoxic agents have been coupled to this peptide to produce prodrugs that are selectively cytotoxic to PSA-producing prostate cancer cells *in vitro* (8, 10, 24, 25). Significant antitumor effects have been observed when these PSA-activated prodrugs have been given to animals bearing PSA-secreting prostate cancer xenografts without producing significant host toxicity (8, 25).

Enzymatically active PSA is found in high levels in the seminal fluid (0.3-5 mg/ml) (11, 26) and in the extracellular fluid of both normal and malignant prostate cancer cells (i.e. 50-500 μg/ml) (15). In contrast, levels of hK2 in the seminal fluid are about 1 % of those of PSA (26) while hK2 levels in the extracellular fluid of prostate cancers have not been reported. Using a chromogenic substrate (i.e. Pro-Phe-Arg-pNa), however, Mikolajczyk et al demonstrated that the enzymatic activity of hK2 was 20,000-fold higher than that of PSA on a comparable substrate containing a tyrosine cleavage site (27). In addition, while PSA and hK2 are both found almost exclusively in the prostate, hK2 is more highly expressed by prostate cancer cells than by normal prostate epithelium. Unlike PSA, hK2 expression appears to increase in more poorly differentiated cancers with the strongest staining observed in prostate cancer lymph node metastases (14). Intensity of staining for hK2 has been found to increase with increasing Gleason grade (14). In contrast, PSA staining tends to decrease with increasing Gleason grade (14). Thus, although hK2 is produced at a lower level than PSA in prostate tissue, the increased production in more poorly differentiated cancers coupled with the several orders of magnitude higher enzymatic activity suggest that total hK2 enzymatic activity in the extracellular fluid may be similar or

even greater than that of PSA. Therefore, hK2 represents an attractive alternative candidate for prostate targeted prodrug activation therapy

Although the hK2 protein is ~ 80% identical to PSA in primary structure (13) , the two are markedly different in their enzymatic properties (11). While PSA is the only member of the kallikrein family with chymotrypsin-like substrate specificity, hK2 displays the trypsin-like specificity of the common to the kallikrein family (13). Like PSA, the biological function and physiologic substrate of hK2 is not fully understood. In vitro, hK2 can cleave the zymogen form of PSA (i.e. pro-PSA) to produce mature, enzymatically active PSA possibly explaining their concerted expression (28, 29). HK2 may also be able to autoactivate its own zymogen form (i.e. pro-hK2) by hydrolysis of the 7 amino acid propeptide leader sequence of hK2 (11, 30). HK2 can cleave fibronectin and protein C inhibitor (11), can activate the single-chain uPA to the active two-chain form uPA (29) and can inactivate plasminogen activator inhibitor-1, the major tissue inhibitor of uPA (31).

Both PSA and hK2 are also involved in the liquefication of the seminal coagulum by proteolysis of the predominant ejaculatory proteins semenogelin I and II (20, 32, 33). Lilja and colleagues determined a specific cleavage map of semenogelins I and II after digestion with hK2 and PSA (20, 32, 33). Like PSA, hK2 can cleave semenogelin proteins at specific sites, but the physiologic role in the cleavage of these proteins is unclear (11). The goal of the present study was to identify specific hK2 peptide substrates that could be utilized to produce prodrugs that are selectively activated by enzymatically active hK2 present in the extracellular fluid of prostate cancer sites. In the present study we have generated random combinatorial peptide libraries in order to rapidly screen a large number of sequences in order to identify putative hK2 substrates. A lead peptide substrate that is efficiently hydrolyzed by hK2 was identified and used to produce an hK2-activated TG prodrug. Since this prodrug must be administered systemically via the blood, pharmacokinetic analyses were performed to determine stability and half-life of the prodrug in vivo.

Materials and methods

Materials: A mutant form of hK2 was utilized for these studies in which the amino acids -5 to -1 of the propeptide sequence of hK2 (i.e.Leu-Ile-Gln-Ser-Arg) were mutated to Asp-Asp-Asp-Asp-Lys to generate a pro-hK2 protein that can be activated to functional hK2 by factor Xa (34). Compared with wild-type hK2, expression of the propeptide hK2 mutant increases the expression levels up to 15-40-fold (34). The generation and characteristics of this mutant hK2 have been previously described (34). Fmoc amino acids were purchased either from Advanced Chemtech or Novabiochem. Reagents were used without further purification. All other reagents were from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified in the text.

Immobilized Peptide Synthesis and Hydrolysis Determination: Peptides ("PepSpots") were provided by Jerini Biotools (Berlin, Germany) and were synthesized on continuous cellulose assays using the SPOT-synthesis technique (35). Each peptide contained an amino-benzoic acid (Abz) moiety at the N-terminus. Abz is a fluorescent molecule with optimal excitation at 325 nm and emission maxima at 420 nm. Peptides were synthesized on cellulose membranes then punched out as small disks into 96-well microtiter plates. Approximately 8 nmoles of peptide are synthesized per spot.

To perform protease assay, spots were rinsed first for 5 minutes with 200 ml of methanol in order to solubilize peptides. Spots were then rinsed 4 times for 10 minutes under gentle agitation with a buffer consisting of 50 mM Tris and 0.1 M NaCl, pH 7.8 (Buffer A). Fresh buffer A was added to each well along with an aliquot of purified protease (i.e. mutant hK2 or trypsin) or 50% human serum in was buffer A. The plate was sealed with plastic and reaction allowed to occur at room temperature without agitation. At described intervals (i.e. 1, 2, 4, 7 and 24 hrs) a 50 ml aliquot of the reaction mixture was transferred to a new 96-well microtiter plate. Fluorescence was then measured at room temperature using a 96-well fluorometric plate reader (Fluoroscan II) with excitation of 355 nm and emission 408 nm. Fluorescence at each point was plotted and reaction rates determined from slope of the best-fit line. Rates are expressed in relative fluorescence units/hr/mg of protease.

Combinatorial Libraries:

Combinatorial peptides libraries were synthesized as described previously (36). Peptides were anchored to the PEGA support resin (Polymerlabs, Amherst MA, 400 microns, 0.2 mmoles/g) proved more successful. without a cleavable linker.

Amino acid couplings were performed according to established Fmoc/tBu protocols using Hobt/DIC activation (37) and performing standard double couplings. Generally, completion of acylation reactions was verified by both Ninhydrin (38) and fluorescamine testing (39). Deprotection of the side-chain protecting group was performed by using Reagent K (TFA/thioanisole/water/phenol/EDT 82.5:5:5:5:2.5 v/v). The Fmoc protecting group was removed with 25% piperidine in DMF. N-α-Fmoc-N-β-t.-Boc-L-diaminopropionic acid (Fmoc-Dpr(Boc)-OH, Novabiochem) was used for the introduction of Dap. Three randomized positions were introduced using a Labmate parallel Organic Synthesizer (4x6 vessels, Advanced Chemtech, Louisville, KY) according to the split-and-mix procedure (40). All natural amino acids, except for cysteine were used with the following side-chain protection: Trt (Asn, Gln, His), tBu (Tyr), OtBu (Asp, Glu, Ser, and Thr), Boc (Lys, Trp) and Pmc (Arg). Amino acid stock solutions (0.5 M with 0.5 M Hobt) were mixed with DIC for 20 min (4 Eq. of each). The activated amino acids were added to the resin and 0.15 ml of 5% DIEA in DMF was added. After 2-3 hrs, the resin aliquots were washed (3 x NMP, 3 x MeOH, 3x DMF) and couplings were repeated with 2 eq. amino acid for 1-2 hrs. A resin sample of each aliquot was subjected to a Ninhydrin and a fluorescamine test which showed completion of the acylation reactions in all cases. Next, the resin aliquots were pooled ($FmocX_1$-X_2-X_3-Dap-Phe-K(Abz)-PEGA) was deprotected with piperidine and the remaining four constant residues, alanine, lysine, glycine and nitrotyrosine (Y', Fluka) were added as Fmoc amino acids in batch with Hobt/DIC activation as described above. For the final deprotection of the side chains, the resin was suspended in Reagent K (1x 10 min, 1x 3 hrs). The resin was washed with 95% acetic acid (3x), DCM (3x), DMF (3x), 5% DIEA in DMF (3x), and DMF (6x). The resin was stored until screening suspended in DMF at -20 °C.

For screening, approximately 1 ml of resin (~ 65'000 beads) was first suspended in methanol in a Petri-dish and examined under transilluminant UV light (302 nm) to detect any falsely positive fluorescent beads prior to addition of protease. After removal of ~ 40-50 beads, the resin was washed with water and finally suspended in 10ml buffer in a glass Petri dish. After a final screen for false-positives, hK2 was added from a frozen stock solution to make 4 μg/ml final. Fluorescent beads were selected and removed with a micropipette, washed with 1 M NaCl, water, DMF, MeOH, water and stored in MeOH at - 20 °C.

Peptide sequencing

Peptide sequencing was completed at the University of Arizona Laboratory for Protein Sequencing and Analyses using an Applied Biosystem 477A Protein/Peptide sequencer (Edman chemistry) interfaced with a 120A HPLC (C-18 PTH column, reverse-phase chromatography) analyzer to determine phenylthiohydantoin (PTH) amino acids.

Automated synthesis of fluorescence-quenched peptides. For validation of the Edman results, peptides were re-synthesized using a Rainin PS3 peptide synthesizer with HBTU/NMM activation. Peptides were synthesized on PEGA resin for on-bead analysis or on Fmoc-Lys(Mtt)-Wang resin for solution assays. The Fmoc-Lys(Mtt)-wang resin was first deprotected with 2% TFA in DCM (3x 2 min). The ε-amine of lysine was then acylated with Boc-Abz. Deprotection/cleavage was performed in TFA/TIS/water (95:2.5:2.5 v/v) for 2-3 hours. Peptides were ether precipitated, dried and purified by C18-HPLC using a linear gradient of acetonitrile (0.1 % TFA), lyophilized and dissolved in DMSO. Peptide identities were confirmed by analysis on a Perseptive Voyager DE-STR MALDI-TOF using dihydroxy benzoic acid as a matrix. Fluorescence measurements were performed on a Fluoroskan II 96-well plate reader (ICN biomedicals, Costa Mesa, CA; excitation, 355 nm; emission, 460 nm). Kinetic parameters were calculated as described earlier (24).

Plasma stability assays

Mouse plasma was obtained from cardiac puncture of anesthetized mice prior to euthanization by CO_2 overdose according to protocols approved by the Johns Hopkins Animal Care and Use Committee. Human plasma was obtained from discarded, pooled, and unlabeled clinical samples. Plasma was diluted to 50% in Tris buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.8). To this, test peptides/prodrugs were added to 250 or 500 μM final concentration. After the incubation period, one volume of acetonitrile was added to precipitate the protein fraction and the tube was centrifuged at 14'000 for 2 min. The supernatant was analyzed by C18-HPLC and the collected peaks were analyzed by MALDI-TOF as described above.

Peptide-prodrug synthesis

The peptide sequence GKAFRRL was synthesized on a Rainin PS3 automated peptide synthesizer on Fmoc-Leu-Wang resin (100 μmoles scale). The same protecting groups were used as during the combinatorial synthesis, except for the lysine, which was orthogonally protected with the ivDde group (Fmoc-Lys(ivDde)-OH, Novobiochem). After deprotection of the N-terminal glycine, the amine was acetylated with acetic anhydride and NMM.

Deprotection of the acid-labile protecting groups and purification was performed as outlined above. Boc-12 ADT was synthesized as previously described (23). TFA treatment, followed by semi-prep HPLC and lyophilisation afforded the amine containing 12ADT. The protected peptide (ac-GK(ivDde)AFRRL) was coupled to 12ADT after Hobt/DIC activation. After completion of the reaction, the ivDde group was removed by adding hydrazine to the reaction mixture (2% final, 30 min). Semi-preparative HPLC yielded acGKAFRRL-12ADT, typically in 60-70% yield. Product was confirmed by MALDI-TOF analysis

Determination of plasma levels of ac-GKAFRR-L12ADT prodrug

Calibration standards consisted of ac-GKAFRR-L12ADT prodrug, RL12ADT or L12ADT spiked into mouse plasma and plasma samples from treated mice were analyzed by liquid chromatography coupled to a quadripole mass spectrometer (LC/MS/MS) [PESciex API 3000]. A multistep gradient elution HPLC method was used to separate the ac-GKAFRR-L12ADT prodrug from the free RL12ADT and L12ADT with eluent A= 1% acetic acid in deionized water and eluent B= 90% acetonitrile/1% acetic acid/deionized water. Samples were eluted through a Zorbax SB-C18 Rapid Resolution column (2/1 x 30 mm, 3.5 μm) at a flow rate of 0.3 ml/min and gradient of 100% A to 100% B over 12 minutes. Calibration was done using standards of ac-GKAFRR-L12ADT added to and then extracted from mouse plasma in a range of 0.001-10 μM, and linear regression analysis was used to generate best-fit lines, from which peak areas of samples were converted to concentration of prodrug. Peak areas of RL12ADT and L12ADT were below limit of detection at all time points and, therefore, calibration was not performed. Single-dose pharmacokinetics were assessed by noncompartmental analysis (41). The area under the curve from time zero to infinity (AUC0-∞) was calculated with the linear trapezoidal method (41). The terminal half-life (T1/2) was determined from the terminal slope (ke) on a log-linear plot of concentration versus time.

Results

Identification of an hK2 Specific Peptide Substrate

The critical requirement for an hK2-activated prodrug is identification of a peptide substrate that is efficiently and selectively hydrolyzed by the enzyme. Therefore, the first task was to define the peptide substrate requirements of hK2. Previously, Lilja et al identified a number of hK2 cleavage sites within the human seminal proteins semenogelin I and II (20). Based on this hK2 cleavage map for SgI and Sg II, small peptides were synthesized containing the amino acid sequence on the N-terminal side of the scissile bond (i.e. P6-P1 where P1 is the amino acid at the cleavage site). These peptides were synthesized using a technique referred to as the SPOT technique (for a recent review, see (35)) in which the C-terminus of the peptide is tethered to a cellulose membrane and the amine of the N-terminus of the peptide is labeled with the fluorophore 2-Aminobenzoic acid (Abz). HK2 digestion releases the Abz containing portion of the peptide and the degree of hydrolysis can thus be monitored by measuring the increase in fluorescence of the supernatant over time.

Using this SPOT technique, the peptides representing hK2 cleavage sites within SgI and II were synthesized and screened for hK2 hydrolysis. Within the group of semenogelin cleavage sites defined by Lilja et al (20), the amino acid leucine was present in the P'1 position in 5 of the 10 sites. Therefore, for this assay, the P'1 position (C-terminal of scissile bond) was fixed as leucine. Glycine was introduced at the amino terminus of all peptides to provide a linker between the Abz and the P6 position of the semenogelin cleavage site peptide sequence. The relative hydrolysis rates of these SgI and II native hK2 cleavage-site peptides were then obtained by incubating enzymatically active hK2 with the cellulose membrane peptides and measuring fluorescent activity released into the supernatant following hK2 hydrolysis of the peptide (Table I). The semenogelin sequences demonstrated a range of hydrolysis rates and not all semenogelin sequences showed significant activity. As previously noted, many of the hK2 cleavage sites within semenogelin protein contain tandem basic amino acid residues at position P1 and P2 with the P1 position being arginine in all but one sequence and the P2 being either arginine, histidine or lysine in 5 of 11 sites (20). In our analysis, the most active native SgI/II sequence (GGKAHRL) contains besides the arginine at P1 two more basic residues (Lysine and Histidine), suggesting a preference for positive charge by hK2 (see table 1).

The occurrence of arginine in the P1 position is a common phenomenon in the natural hK2 substrates (11). Therefore, in an attempt to produce better hK2 substrates, the native sequences (Table 1, bold) were modified by substituting the amino acid in the P2 position for arginine, thereby producing an R-R cleavage site. In each case substitution of R-R for the native P2-P1 sequence resulted in peptides that were for the most part better (i.e. 1.2 to 74-fold) hK2 substrates. Next, on the basis of hK2 hydrolysis rates, the two best substrates (i.e. GHEQKRRL and GGGKARRL) were subjected to further sequence modifications that included substitution of other amino acids in the P'1 position, substitution with *d*-amino acids, and substitution of alanine in each position (i.e. Alanine screen) (data not shown). From these experiments, it could be concluded that at the P'1 position, Leucine is preferred over Aspartic acid and Serine (data not shown). Substitution of *l*-arginine over *d*-arginine in P1 and/or P2 markedly decreased activity (i.e. dR-R= 4.4-fold; R-dR= 43-fold; dR-dR=130- fold decreased activity), whereas substitution of d-leucine in the P'1 position only decreased activity ~2-fold (data not shown).

To determine if there was a strict requirement for arginine in the P1 position of the SgI/II-based peptide sequences, additional peptides were synthesized with histidine substituted for arginine in the P1 position, table 1. In all cases these P1 histidine containing substrates were markedly poorer substrates for hK2 hydrolysis, table 1. In additional studies, soluble fluorescence quenched peptide substrates based on the the SgI/II sequence GSKGHF**R**L were produced in which the P1-arginine was substituted with the positively charged amino acid lysine (i.e. GSKGHF**K**L and GSKG**P**F**K**L). The arginine-free sequence GSKGHFHL, identified as native substrate from the SGI/II hK2 cleavage map ()was also synthesized for testing in solution. In these studies, none of these three arginine free peptides were appreciably digested by hK2, even after prolonged incubation. These results further support results from earlier studies using small peptide substrates and phage display and demonstrate that hK2 has a strong preference for arginine in the P1 position of peptide substrates. Overall, from these studies it appeared that hK2 prefers polar, positively charged substrates with a monobasic or dibasic RR motif at the cleavage site.

Finally, to determine whether these SGI/II-based peptide substrates were selective for hK2 hydrolysis, each sequence was incubated with equimolar amount of trypsin. A comparison of hydrolysis rates for each individual peptide for hK2 vs. trypsin demonstrated that all of these peptides were better substrates for trypsin, table 1. HK2 hydrolysis rates or this series of peptides ranged from 1% to 78% of trypsin hydrolysis rates.

On the basis of these studies, the GKAFR peptide was selected for further analysis based on high relative hK2 vs. trypsin hydrolysis rates. A fluorescent substrate was synthesized by coupling the fluorophore 7-amino-4-methyl coumarin (AMC) to the carboxy-terminus of the peptides to produce the substrate Mu-GKAFR-AMC (where Mu is Morpholinocarbonyl). Rates of hydrolysis by hK2 and trypsin were determined and compared to the kallikrein substrate PFR-AMC. In addition, stability to non-specific hydrolysis in human plasma was also assayed, table 2. These results demonstrate that the

GKAFR-AMC substrate is a better substrate for hK2 than PFR-AMC, however, neither substrate was selective for hK2 hydrolysis nor were these substrates stable to hydrolysis in human plasma, table 2.

Screening a combinatorial peptide library to identify hK2 substrates

Trypsin and trypsin-like proteases have a defined preference for the amino acid arginine or lysine at the site of hydrolysis. There are a large number of trypsin-like proteases present in human blood, including human kallikrein 1, plasmin, thrombin, and other members of the clotting factor cascade. The proteolytic activity of these proteases in the blood is tightly regulated and these proteases are present in the bloodstream predominantly as inactive zymogens. HK2 is also a trypsin-like protease with a preference or, perhaps, a requirement, for arginine at the P1 hydrolysis site (21). As our experience with the peptide sequences defined above indicated, peptide substrates containing arginine appeared relatively unstable to non-specific hydrolysis in the blood. Therefore, in an attempt to identify more selective hK2 peptide substrates and to determine if hK2 indeed has a strict requirement for arginine at site of cleavage, a fluorescent-quenched combinatorial peptide library was synthesized containing random amino acid in positions P3-P1 to allow for screening of all possible amino acid combinations (i.e. 19^3 or 6859 sequences).

Previously Meldal and co-workers demonstrated that protease substrate requirements can be routinely mapped by on-bead (i.e. resin) digestion of short peptides (36). By following the 'split-and mix' approach (40), a peptide library is generated on polymeric solid-phase synthesis resin "beads" so that each bead contains at the end a unique but random peptide sequence. These peptides are bracketed by a fluorophore at the C-terminus [2-amino benzoic acid (Abz) coupled to the ε-amino group of lysine] and a pairing quencher moiety at the N-terminus (3-nitro tyrosine). Upon hydrolysis of any backbone amide bond, the quencher-containing N-terminal part of the peptide is liberated and diffuses into the solution, resulting in bright fluorescence due to un-quenching of the remaining C-terminal part, still linked to the bead (36). The polymeric support has to swell sufficiently in water to allow diffusion of the protease into the bead. Our previous data, using the SPOT-based peptides suggested that the positively charged tripeptide GKA would be a close-to-optimal P6-P4 amino acid sequence and that the C-terminal positions were of more significance for defining selectivity of hK2 activity. Therefore, this library was biased in that, between the N- terminal lysine-ABZ fluorophore and the C-terminal nitrotyrosine quencher a constant tripeptide (GKA) was inserted in positions P6-4 followed by random amino acids in positions P3-P1 (i.e. GKAXXX). PEGA (a mix of polyacrylamide and polyethylene glycol) resin was chosen as the solid phase support based on preliminary studies demonstrating superiority of this resin over alternative resin supports (e.g. TentaGel) for this application (data not shown).

Additionally, Walle and co-workers (42) published data from a small combinatorial library to find linkers for the optimal display of peptide ligands to various protein targets. They reported that the insertion of a dipeptide consisting of a cationic residue together with a hydrophobic residue presents a general method for optimizing peptide display on solid phase beads. To test this observation in our own system, we incorporated a linker described by Walle et al in which the cationic residue was diamino propanoic acid (Dap) and the hydrophobic residue was phenylalanine (F). We synthesized the test peptide Y'GKAFRL-**Dap-F**-K'-PEGA and observed that the time required to generate clearly detectable fluorescence on the beads was reduced to 4-5 hours compared to 10-12 hours for the same peptide sequence lacking the Dap-F linker. On the basis of these results, the Dap-F dipeptide linker was included in all subsequent libraries.

Therefore, the final library used for screening with hK2 contained the general sequence Y'GKAXXX-Dap-F-K' PEGA where X = any of 19 amino acids (cysteine was excluded from library) and contained 19^3 peptide sequences on ~ 50,000 beads (i.e. ~ 7 beads for each unique peptide sequence). After carefully removing any false positive fluorescent beads from the library (~ 40-50 beads), purified, enzymatically active hK2 was added at a final concentration of 4 μg/ml. After 1 hour, the first positive bead was removed. Over the subsequent 3 hours, 9 more beads were selected. In total, 14 beads were selected over a period of 24 hours. Positive beads were sequenced by Edman degradation.

Seven out of fourteen peptides contained one or more arginine residues. The peptides lacking any arginine did not show a specific amino acid preference. One sequence (FRR) was similar to double arginine motif sequences previously identified as optimal in the SPOT assay.

In order to confirm that the selected sequences represented true hK2 substrates and not false positives, the majority of the fluorescence quenched peptides were re-synthesized, cleaved from the resin and tested for hydrolysis by hK2 in solution. After re-synthesis, none of the soluble non-arginine containing peptides were hydrolyzed by hK2 (data not shown), confirming that the arginine-free sequences were not hK2 substrates but false positives. In contrast, each of the re-synthesized arginine containing peptide substrates were readily hydrolyzed by hK2. Our combinatorial screen, identified seven arginine-containing peptides. Four of these were re-synthesized (X1-X2-X3 = RAF, KPR, FRR and MRQ respectively). Three other lead sequences were not re-synthesized (X1-X2-X3 = IQR, FRK and VRQ respectively). All four arginine-containing sequences that were re-synthesized reproduced fluorescence when these peptides were digested on-bead with hK2. For a more quantitative analysis, the fluorescence-quenched peptides were cleaved off the resin and purified by HPLC. The rate of hydrolysis was quantified by measuring increase in fluorescence, figure 2. The best substrate proved to be the sequence with arginine at P1 and P2 (i.e. Y'GKAFRR-Dap-F-K'). In less than five minutes, more than 50% of the peptide was digested (500 μM peptide, 4 μg/ml hK2). For the other peptides, digestion of the same amount of peptide took 19-29 minutes. Maximum digestion never exceeded more than 70-75%, a value that was reached with Y'GKAFRR-Dap-F-K' in less than 15 minutes. In subsequent studies, hydrolysis rates using the Y'GKAFRR-Dap-F-K' peptide were analyzed by Lineweaver-Burke reciprocal plots. The Michaelis-Menten constant (K_m) was determined at 26.5 μM, the k_{cat} at 1.09 sec^{-1} and the k_{cat}/K_m ratio was 41,132 sec^{-1} M^{-1}. These results compare favorable to those previously reported for the Pro-Phe-Arg-AMC substrate used to assay hK2 activity (K_m 40 μM; k_{cat} 0.92 sec^{-1}; k_{cat}/K_m 22,916 sec^{-1} M^{-1}) and were superior to the GKAFR-AMC substrate we generated based on results of SPOT analysis (K_m 146 μM; k_{cat} 0.13sec^{-1}; k_{cat}/K_m 895 sec^{-1} M^{-1}).

Stability of hK2 peptides in plasma

Arginine-containing peptides are potential substrates for the wide variety of other trypsin-like proteases that are present in the blood and may have residual activity in the blood. The plasma stability of an hK2 peptide substrate may therefore be limited and this would have significant consequences related to the development of an hK2 activated prodrug. Therefore, two fluorescence quenched hK2 peptide substrates peptides were incubated in 50% mouse or human plasma (diluted in PBS buffer) to determine stability using a plate-reader. Similar to results with arginine containing peptides from the SPOT analysis, none of these arginine-containing, fluorescence quenched hK2 peptide substrates identified from the combinatorial screening were stable in human or mouse plasma (data not shown). Fluorescence-quenched Y'GKAFRR-Dap-GK' and Y'GKAFRRLGK' (500 μM each) were hydrolyzed when incubated in 50% mouse or human plasma (figure 3). Mouse plasma degraded the peptides faster than human plasma (figure 3). The Leucine containing peptide was less stable than the Dap containing peptide in both plasma types. HPLC analysis of the peptides after 3 hours of incubation confirmed that the fluorescence generated during this plate reader assay corresponded with proteolysis and, in each case, several degradation products were generated; almost no parent peptide remained after 3 hours in mouse plasma (~ 5%) whereas in human plasma, ~25% of intact peptide remained after 3 hours. Overnight incubation in human plasma resulted in complete degradation of both peptides. To determine whether acetylation of the N-terminus of the hK2 peptide would enhance stability, the acetylated fluorescence-quenched peptide, ac-Y'GKAFRRLGK', was synthesized and plasma stability was compared with that of the non-acetylated peptide (Y'GKAFRRLGK'). Hydrolysis of these two peptides was completely identical as judged from the generation of fluorescence in the plate-reader assay and by HPLC analysis. These results indicate that non-specific hydrolysis of the hK2 peptide substrate is not due to degradation by plasma aminopeptidases.

Synthesis and characterization of an hK2 activated thapsigargin prodrug

The preceding results would suggest that the development of an hK2 activated peptide-based prodrug might not be feasible due to poor stability of arginine containing peptides in plasma. However, the possibility remained that the introduction of a bulky hydrophobic moiety like an analog of thapsigargin might alter the relative rate of hK2 hydrolysis and/or stability in plasma. Therefore, we proceeded to synthesize a putative hK2-activated prodrug by coupling the GKAFRR peptide sequence to a primary amine containing TG analog. In previous studies we had identified a potent amino acid containing TG analog [i.e. 8-O-(12-[L-leucinoylamino]dodecanoyl) -8-O-debutanoylthapsigargin (L12ADT)] that was as cytotoxic as TG with an LD_{50} value of ~30 nM against human prostate cancer cells *in vitro* (23) . Previously, the L12ADT analog has been coupled to a PSA-specific peptide to produce a prodrug that is selectively cytotoxic to PSA-producing prostate cancer cells in vitro and in vivo (8).

On the basis of these results the prodrug ac-GKAFRR-L12ADT was synthesized. This putative hK2 prodrug was incubated with enzymatically active hK2 (4 μg/ml) to determine extent of hydrolysis over time. HPLC analysis of aliquots of the incubation mixture indicated that the hK2 prodrug is rapidly cleaved by hK2 (Figure 5). MALDI-TOF analysis of the digestion products indicated that cleavage occurred after each arginine residue generating both Arg-Leu-12ADT (RL-12-ADT) and L-12ADT. In 25 min, 50% was hydrolyzed and after 1 hour, more than 80% of the starting prodrug was hydrolyzed, figure 5. The ratio of the products RL-12ADT : L-12ADT was 1: 1.8, as determined by HPLC integration. In previous studies the IC50 of L12ADT against human TSU bladder cancer cell line was ~ 30 nM. RL-12ADT was ~ 5-fold less potent against this cell line in growth inhibition assays in vitro (data not shown).

To determine the plasma stability for the hK2 prodrug, we incubated the prodrug in 50% fresh heparinized (1%) plasma for 24 hours at room temperature, precipitated the proteins by adding one volume of 1% TFA in acetonitrile and analyzed the supernatant by HPLC. Unexpectedly, HPLC analysis after 24 hrs incubation in plasma yielded only a single peak corresponding to the hK2 prodrug. MALDI-TOF MS of the isolated single peak confirmed the plasma stability of the prodrug.

hK2 levels in prostatic tissues and human prostate cancer cell lines

The levels of hK2 production in benign and malignant prostate tissue have not been as well characterized as PSA levels. In a previous study, Lovgren et al demonstrated that the average hK2 levels in the seminal plasma was 6.4 μg/ml (26). In this study, the level of PSA in the seminal plasma was 820 μg/ml (26). We have isolated prostatic fluid taken directly from radical prostatectomy specimens (i.e. without contamination from seminal vesicle fluid) and measured PSA and hK2 levels. In this prostatic fluid, PSA levels were 696 ± 305 μg/ml (n=4) while hK2 levels were 165-fold lower at 4.2 ± 0.5 μg/ml (n=5). Haese et al evaluated preoperative PSA and hK2 levels in men with stage T2 or T3 prostate cancers undergoing radical prostatectomy (47). Men were selected with PSA values < 10 ng/ml. In this study the PSA level for men with T2 vs. T3 was not significantly different and was approximately 6 ng/ml. In contrast, hK2 levels in the serum of these patients were 50- (stage T3) to 75- (stage T2) fold lower (i.e. 0.08-0.12 ng/ml respectively (47).

In contrast, hK2 levels in conditioned media of human prostate cancer cell lines are several orders of magnitude lower (i.e. 0.1-60 ng/ml after 4 days conditioning). For example, in one experiment, 6 x 10^6 LNCaP human prostate cancer cells produce hK2 levels in the conditioned media of ~ 16.7 ng/ml while PSA levels were 166 ng/ml after 4 days conditioning (data not shown). Thus, in vitro assessment of hK2 selective cytotoxicity of an hK2-activated prodrug is problematic because the available cell models produce much lower levels (i.e. ~ 100-fold) of hK2 than estimated levels in extracellular fluid of human prostate cancers.

Cytotoxicity of hK2-activated prodrug in vitro

To determine the in vitro efficacy and selectivity of the hK2-activated prodrug, cytotoxicity against a series of hK2-producing cell lines (i.e. CWR22R, LNCaP, C4-2B) was compared to cytotoxicity against TSU a non-hK2 producing human bladder cancer cell line, figure 6. In previous

study the levels of hK2 production by these human prostate cancer cell lines was determined (48). The lowest levels were found in the CWR22R line which produced 1.1 ± 0.2 ng hK2/10^6 cells/day (48). LNCaP cells produced 2.2 ± 0.6 ng hK2/10^6 cells day and C4-2B cells produced 14.9 ± 3.0 ng hK2/10^6 cell/day, the highest level of all the cell lines tested (48). In this experiment, the hK2 prodrug had similar inhibitory effect on cell growth after 7 day exposure at concentrations ≥ 1.25 μM in all cell lines tested, figure 6. A modest difference in effect was observed at for all hK2 producing cell lines at lower concentrations of prodrug. C4-2B cells, the line that produces highest levels of hK2, appeared to be the most sensitive to the prodrug, figure 6. The estimated IC_{50} for TSU in this study was ~1.25 μM while the IC50 for the highest hK2 producing line, C4-2B was ~0.3 μM.

The modest ~4-fold difference in activity between an hK2-producing vs. non-producing cell line in this experiment may be secondary to production of low levels of hK2 by these human prostate cancer cell lines. Therefore, to determine the relative efficacy and specificity of an hK2-activated prodrug against requires conditions that more closely mimic levels of enzymatically active hK2 found in extracellular fluid of human prostate cancers. In previous studies, we demonstrated that PSA levels in extracellular fluid of human prostate cancers was 69 ± 12 μg/ml (15). However, hK2 levels in the extracellular fluid of human prostate cancers have not been reported. To determine approximate levels of hK2 in relation to PSA we measured hK2 and PSA levels in the human PC-82 prostate cancer xenograft model. PC-82 does not grow as a cell line in vitro and is maintained through serial passage in nude mice. In previous studies we determined that PSA levels in PC-82 xenografts were only ~3-fold lower than those found in human prostate cancers (15). In contrast, in LNCaP xenografts the levels of PSA were ~ 45-fold lower than those observed in human prostate cancers (45). Therefore, the PC-82 represents a more relevant model for estimating levels of hK2 in human prostate cancers. PC-82 xenografts were harvested and homogenized and then total PSA and hK2 levels in tumor lysates were determined by ELISA assay (Hybritech). The concentration of PSA in these PC-82 lysates was 5.15 ± 0.35 μg/g of tissue while the levels of hK2 were ~12-fold lower at 0.41 ± 0.06 μg/g of tissue.

On this basis we estimated that a level of hK2 of ~1 μg/ml in the conditioned media would roughly approximate levels of enzymatically active hK2 found in extracellular fluid of human prostate cancer xenografts. Therefore, in the next series of experiments, non-hK2 producing human TSU cells were treated in serum containing media ± 1 μg/ml purified, enzymatically active hK2. Clonal survival assays were then performed after 5 day exposure to hK2-activated prodrug, figure 7. In these experiments there was an ~ 10-fold enhancement of efficacy (i.e. IC50 ~ 0.5 μM in the presence of hK2 vs ~ 5 μM in the absence of hK2) of the hK2-activated drug in the presence of 1 μg/ml enzymatically active hK2 in the serum containing tissue culture media.

Pharmacokinetics and toxicity studies

Prior to performing antitumor efficacy experiments we needed to establish a dose that would be tolerated with minimal toxicity to the animal. To accomplish this, Balb-C mice were treated in groups of 3 with a single intravenous injection of increasing doses of the ac-GKAFRR-L12ADT prodrug to establish the dose that killed 100% of mice (i.e. LD_{100}). In these studies the LD_{100} was determined to be 11 μmoles/kg (i.e. 18.2 mg/kg). All mice, however, tolerated a single intravenous dose of 3.67 μmoles/kg and this dose was then used for further dosing and pharmacokinetic studies. An additional group of mice (n=8) received five consecutive daily intravenous injections with 3.67 μmoles/kg prodrug without any deaths or observable toxicity (i.e. weight loss < 15% over baseline).

To determine pharmacokinetic parameters for the hK2-activated thapsigargin prodrug, Balb-C mice (n=3/timepoint) were treated with a single intravenous dose of 3.67 μmoles/kg of the ac-GKAFRR-L12ADT prodrug, figure 8. At various time points (5, 10, 30 minutes and 1, 1.5, 2, 3, 4, 6, 12, 24 hrs) mice were sacrificed after blood was obtained by cardiac puncture. After precipitating serum proteins with acetonitrile, supernatants were evaluated by LC-MS to determine concentrations of ac-GKAFRR-

L12ADT, R-L12ADT and L12ADT at each time points. Areas under the curve were converted to concentrations based on a standard curve that was linear for concentrations ranging from 1 nM to 10,000 nM. In this study, the Cmax occurred at 10 minutes post injection and was 36.8 $\pm$ 7.2 μM, figure 8. The half-life of the prodrug was 40.7 $\pm$ 1.2 minutes and the area under the curve was 2444.8 $\pm$ 39.1 μmol*min/l, figure 8. Both RL12ADT and L12ADT were below the lower limit of detection (i.e. < 1 nM) for all time points, figure 8. On the basis of these studies, we concluded that the ac-GKAFRR-L12ADT prodrug is highly stable to hydrolysis in the serum in vivo.

Hydrolysis of hK2 peptide and prodrug by other potential tumor-associated proteases

Due to their short length, the hK2 peptide substrates could potentially be substrates for other trypsin-like proteases as well. Although a rather specific protease substrate can be defined with 7 amino acids, there is a lack of higher order structural information by which natural protein substrates normally impose high specificity. Cancer progression is often correlated with increased protease activity (43). These activities could be potentially beneficial since they could broaden the scope of applications for protease activated prodrugs. To test the hypothesis that other tumor-associated proteases could activate the ac-GKAFRR-L12ADT prodrug, we selected a number of known proteases (table 3), implicated in cancer progression to determine if our lead substrate, GKAFRRL could be efficiently hydrolyzed by any of these proteases. For this analysis, we selected trypsin-like serine proteases plasmin and urokinase and cathepsins B and D. We analyzed hydrolysis of both the fluorescence quenched peptide substrate and the TG-prodrug. No appreciable hydrolysis of either substrate was observed following incubation with Cathepsin D or Cathepsin B. Urokinase showed low activity on the fluorescence quenched peptide substrate, but not on the prodrug (table 3). Plasmin had a more than 10-fold slower rate of hydrolysis of the peptide substrate than hK2. However, with the prodrug, plasmin had an approximately 6 –fold higher hydrolysis rate than hK2. Analysis of the cleavage products demonstrated that with plasmin, proteolysis occurs between the two arginines, generating the less potent cytotoxin Arg-Leu-12 ADT. Plasmin, therefore, could be a valid target for selective activation of the GKAFRR-L12ADT prodrug in other types of cancer where plasmin activation may play an important role. Further studies are underway in our laboratory to characterize in vitro activation of the GKAFRR-L12ADT prodrug using non-prostate cancer cell lines that display increased plasmin activity.

Discussion

The peptide substrate requirements of hK2 have been characterized in order to identify peptides that are readily cleaved by this trypsin-like serine protease that could be used to produce prodrugs that are targeted to hK2 producing prostate cancers. Previous to our studies, several groups have evaluated the substrate requirements for hK2. Mikolajczyk et al used a small number of peptide substrates and concluded that hK2 had P1 arginine restricted specificity (21). More recently, Cloutier et al recently used phage display technology to study the substrate specificity of hK2 using large numbers of peptide sequences and also reported that hK2 has a strict preference for arginine in the P1 position (44). Substitution of the P1 arginine with lysine or histidine normally abolished all hK2 hydrolysis. Despite many attempts, including a combinatorial peptide library screen, we also did not identify an arginine-free hK2 substrate, confirming the findings of these previous investigators. We further demonstrated that hK2 prefers dibasic Arg-Arg at the P1-P2 positions, an observation that was initially reported by Lovgren et al. Additionally we observed that substitution of D-Arginine in the P1 and/or P2 position significantly decreases hK2 hydrolysis.

Previously, several studies have demonstrated that inactivation of peptide hormones such as vasopressin as well as major clotting factors occurs by cleavage after arginine by proteolytic activity present in plasma (45, 46). Because these hK2-selective peptides will be incorporated into prodrugs that will need to be administered systemically via the blood, our main concern was that arginine containing peptide substrates for a trypsin-like protease like hK2 would have limited stability in plasma. This would severely limit the feasibility of an hK2 targeted prodrug. Our initial results were discouraging in that all

of the arginine containing hK2 fluorescence quenched peptide substrates were rapidly hydrolyzed in non-hK2 containing human and mouse plasma. However, the L12ADT containing hK2 prodrug was completely stable in human and mouse plasma, possibly due to binding of this prodrug to serum proteins making it inaccessible to active plasma proteases. Besides stability in plasma, the hK2 prodrug ac-GKAFRR-L12ADT was also stable to hydrolysis in mouse plasma in vivo with no appreciable conversion to cytotoxically active RL12ADT or L12ADT over a 24 hr period. In addition, after a single intravenous injection, the LD_{100} was 11 μmoles/kg. These toxicity results are comparable to those we observed previously using a prodrug consisting of a PSA-selective peptide substrate coupled to L12ADT (8). Currently, studies are underway in our laboratory to elucidate the mechanisms for this paradoxical plasma stability of the prodrug vs. the unconjugated peptide substrates.

A number of laboratories are currently developing protease targeted prodrugs using similar types of combinatorial or phage-based screens to identify peptide substrates. Our results demonstrate that the peptide carrier may behave differently when unconjugated to drug than the subsequent prodrug in terms of hydrolysis rates and stability in plasma. This difference most likely is due to certain characteristics (e.g. hydrophobicity, molecular weight, degree of binding to serum proteins) of the drug being targeted. Therefore, the pre-screening of lead peptides unconjugated to drug for plasma stability may not be useful for predicting plasma stability of the subsequent prodrugs.

In this study there was only a ~4-fold difference in cytotoxicity between the highest hK2 producing cell line, C4-2B and a non-hK2 producing cell lines, TSU. This limited differential cytotoxicity most likely is due to the low level of hK2 production by the human prostate cancer cell lines evaluated in this study (48). In this regard, when non-hK2 producing TSU cells were incubated in the presence of higher levels of hK2 that are more representative of levels that may be present in extracellular fluid of human prostate cancers, the differential cytotoxicity increased to 10-fold. Additionally, Kumar et al demonstrated that, in serum containing conditioned media from LNCaP cells, most of the hK2 produced was present as enzymatically inactive pro-hK2, whereas, if these cells were grown in serum free media supplemented with androgen, all of the hK2 was present as mature hK2 (49). Finally, enzymatically active hK2 rapidly forms complexes with serum protease inhibitors such as alpha-2-macroglobulin, alpha 1-antichymotrypsin and alpha 2-antiplasmin, and protein- c inhibitor (20, 21). Thus, in vitro and in vivo assessment of hK2 selective cytotoxicity of the hK2-activated prodrug will be difficult because the available cell models produce much lower levels (i.e. ~ 100-fold) of enzymatically active hK2 than estimated levels in extracellular fluid of human prostate cancers which, in serum containing media may be enzymatically inactive either through lack of proper processing (i.e. pro-hK2) or through binding to serum protease inhibitors. Therefore, to better assess cytotoxicity of this hK2-activated prodrug in vitro and in vivo we are currently measuring levels of hK2 in the extracellular fluid of human prostate cancers in order to generate human prostate cancer cell lines that produce similar levels of enzymatically active hK2.

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Figure legends.

Figure 1: Chemical structure of fluorescence-quenched combinatorial 'one bead-one peptide' library (Y'GKAXXX-Dap-F-K'-PEGA, where Y' is nitrotyrosine and K' is 2-aminobenzoic acid substituted lysine).

Figure 2: Hydrolysis of various lead substrates (250 mM) by hK2 (8 mg/ml) in PBS buffer. Note that the double arginine substrate (Y'GKAFRR-Dap-FK') by far exceeded all other substrates. A substrate without arginine (Y'GSKGHFKL-Dap-F-K') did not show any hydrolysis. To determine 100 % digestion, trypsin was added (50 mg/ml) and samples were incubated to 37°C for 30 min and the fluorescence was determined.

Figure 3: Hydrolysis of hK2 peptide substrates in plasma. Arginine containing lead hK2 substrates (500 mM) were incubated in 50% mouse or human plasma. Generation of fluorescence indicated that the fluorescence-quenched peptides are unstable in plasma. Hydrolysis of the substrates was confirmed by HPLC. Comparison of mouse and human plasma for the same substrate suggest higher proteolytic activity in mouse plasma compared to human plasma.

Figure 4: Chemical structure of hK2 prodrug, acGKAFRRL-12ADT. HK2 cleavage sites are indicated. The ratio of RL-12ADT : L-12ADT generated by hK2 digestion was 1 : 1.8.

Figure 5: HK2 mediated hydrolysis of various peptide and prodrug substrates (125 mM each). Fluorescent substrates were analyzed by means of a fluorescence plate-reader (ex = 355 nm, em = 460 nm). acGKAFRRL-12ADT and acGKAFRRLG were analyzed by HPLC and quantified by HLPC integration.

Figure 6: Cytotoxicity of ac-GKAFRRL-12ADT against hK2-producing CWR22R, LNCaP and C4-2B human prostate cancer cell lines and hK2 non-producing TSU human bladder cancer cell line. Cell lines were incubated in presence of indicated concentration of prodrug for 7 days at which point MTT assay performed to determine viable cell number. Data presented as percent growth inhibition ± standard error compared to vehicle treated controls from each cell line. Data represents results from 8 replicate wells and experiments performed in duplicate.

Figure 7: Cytotoxicity of ac-GKAFRRL-12ADT in the presence of hK2 containing media. Human TSU bladder cancer cells were exposed for 5 days to indicated concentrations of prodrug in either standard serum containing media or same media + enzymatically active hK2 (1 μg/ml). Clonal survival was then compared to survival of vehicle treated controls.

Figure 8: Pharmacokinetics of ac-GKAFRRL-12ADT in Balb-C mice. Mice were given single intravenous injection of 3.67 μmoles/kg of prodrug. At indicated times, blood was obtained from anesthetized animals and prodrug extracted from isolated plasma. HPLC analysis was performed and plasma concentrations of prodrug determined by comparison to standard curve. Data represent mean ± standard error of 3 animals at each timepoint.

Table 1: Relative hydrolysis rates of hK2 substrates by hK2, as determined by release of N-terminal conjugated 2-amino benzoic acid.

	P7	P6	P5	P4	P3	P2	P1	P'1	Type	hK2 rate (FU/hr/ug)	trypsin rate (FU/hr/ug)	Ratio hK2/trypsin
ABZ	G	**G**	**G**	**K**	**A**	**H**	**R**	L	native sgl/II	7.52	17.00	0.44
ABZ	G	G	G	K	A	R	R	L	dibasic RR	8.77	11.20	0.78
ABZ	G	G	G	K	A	H	H	L	His Subst	0.13	18.60	0.01
ABZ	G	**H**	**E**	**Q**	**K**	**G**	**R**	L	native sgl/II	1.19	39.60	0.03
ABZ	G	H	E	Q	K	R	R	L	dibasic RR	10.94	22.20	0.49
ABZ	G	H	E	Q	K	G	H	L	His Subst	0.01	7.20	0.00
ABZ	G	**K**	**D**	**V**	**S**	**G**	**R**	L	native sgl/II	0.45	12.40	0.04
ABZ	G	K	D	V	S	R	R	L	dibasic RR	4.25	11.90	0.36
ABZ	G	K	D	V	S	G	H	L	His Subst	0.12	11.30	0.01
ABZ	G	**P**	**A**	**H**	**Q**	**D**	**R**	L	native sgl/II	0.14	13.20	0.01
ABZ	G	P	A	H	Q	R	R	L	dibasic RR	7.36	18.20	0.40
ABZ	G	P	A	H	Q	D	H	L	His Subst	0.09	0.60	0.15
ABZ	G	**S**	**K**	**G**	**H**	**F**	**H**	L	native sgl/II	0.31	11.00	0.03
ABZ	G	S	K	G	H	R	R	L	dibasic RR	4.86	16.30	0.30
ABZ	G	S	K	G	H	F	R	L	Arg Subst	5.55	11.40	0.49
ABZ	G	**S**	**N**	**T**	**E**	**K**	**R**	L	native sgl/II	0.47	22.60	0.02
ABZ	G	S	N	T	E	R	R	L	dibasic RR	1.09	18.10	0.06
ABZ	G	S	N	T	E	K	H	L	His Subst	0.15	13.30	0.01
ABZ	G	**S**	**Q**	**N**	**Q**	**V**	**R**	L	native sgl/II	0.13	18.62	0.01
ABZ	G	S	Q	N	Q	R	R	L	dibasic RR	3.85	7.80	0.49
ABZ	G	S	Q	N	Q	V	H	L	His Subst	0.06	0.50	0.12
ABZ	G	**S**	**Y**	**E**	**E**	**R**	**R**	L	native sgl/II	0.60	24.20	0.02
ABZ	G	S	Y	E	E	R	H	L	His Subst	0.08	14.60	0.01
ABZ	G	**S**	**Y**	**P**	**S**	**S**	**R**	L	native sgl/II	2.67	10.90	0.24
ABZ	G	S	Y	P	S	R	R	L	dibasic RR	3.78	17.30	0.22
ABZ	G	S	Y	P	S	S	H	L	His Subst	0.05	0.50	0.10
ABZ	G	P	L	I	L	S	R	L	PSA-Propeptide	0.60	6.00	0.10

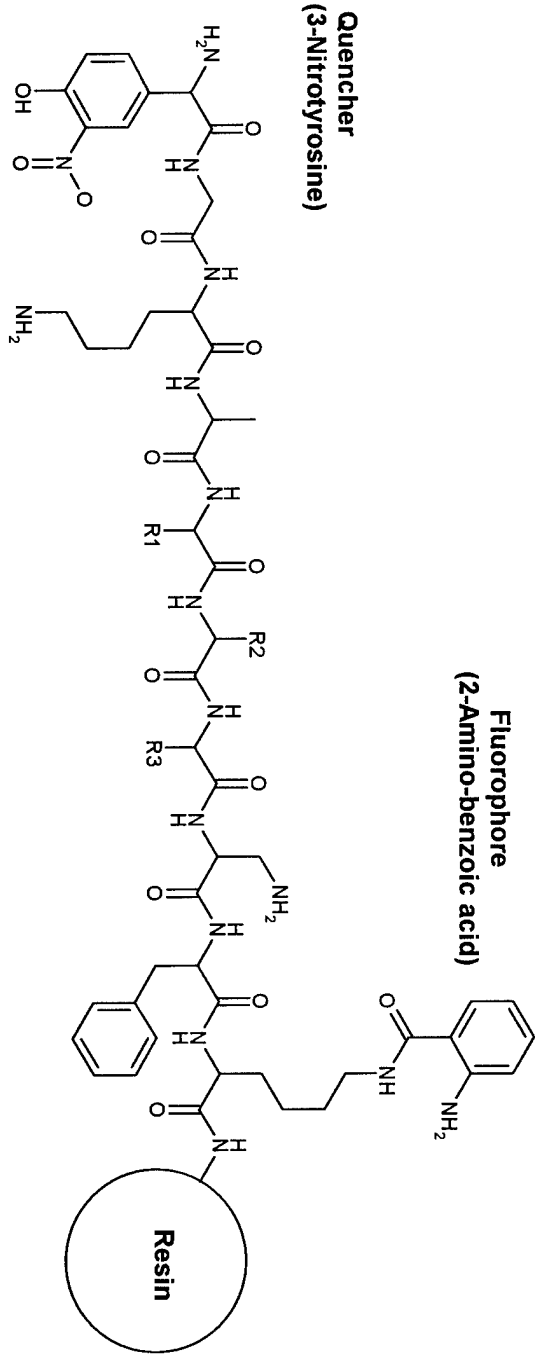
Table 2: Relative hydrolysis rates of hK2 substrates by hK2 and trypsin, as determined by release of N-terminal 7-amino-4-methyl coumarin.

	hK2 (5μg/ml)		trypsin (5μg/ml)		50% Plasma
Substrate	pmol AMC/min	pmol AMC/min/ nmol protease	pmol AMC/min	pmol AMC/min/ nmol protease	pmol AMC/min
Mu-GKAFR-AMC	28.5	1584	1356	85300	28.3
PFR-AMC	15.6	867	436	27400	16.6

Table 3: Relative hydrolysis rates normalized to hydrolysis rate of cathepsin B. Enzyme concentrations were 1 μg/ml. Concentration Abz-GKAFRRLY' was 500 μM, concentration hK2 prodrug (acGKAFRRL12ADT) was 100 μM. A relative hydrolysis rate of 1 corresponds to approximately 0.1 % digestion in 1 hour.

	Relative hydrolysis rate	
Protease	Abz-GKAFRRLY'	hK2 prodrug
Cathepsin B	1	1
Cathepsin D	1	1
Plasmin	36	750
hK2	353	125
Urokinase	17	1

Figure 1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Fluorophore
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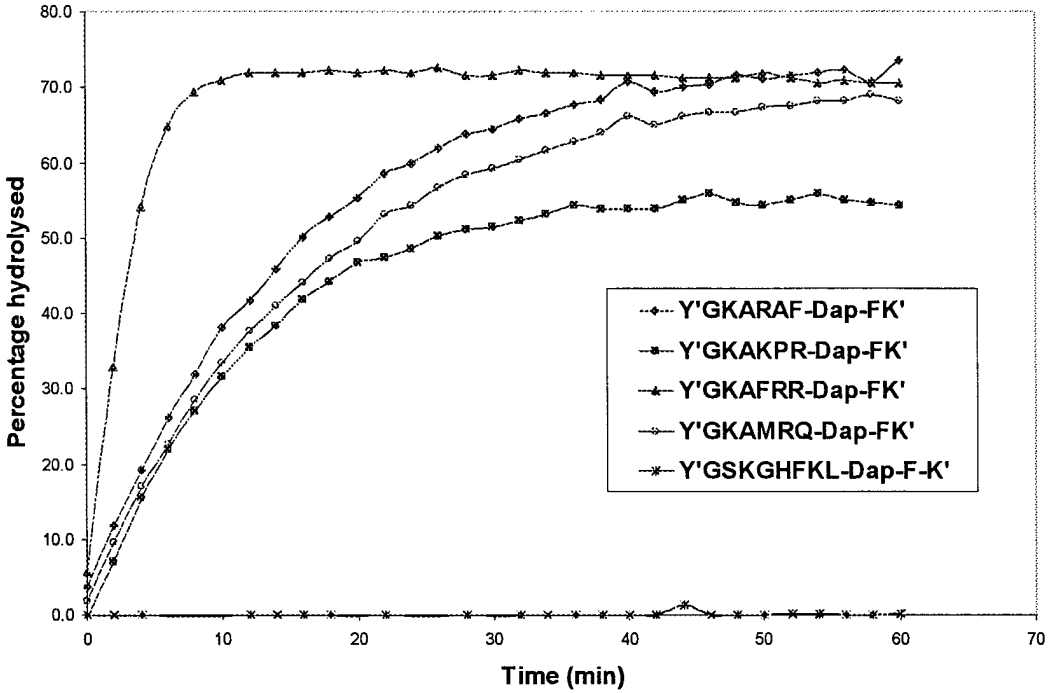
Resin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70.0
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50.0
40.0
30.0
20.0
10.0
0.0
Percentage hydrolysed
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20
30
40
50
60
70
Time (min)
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Y'GKAKPR-Dap-FK'
Y'GKAFRR-Dap-FK'
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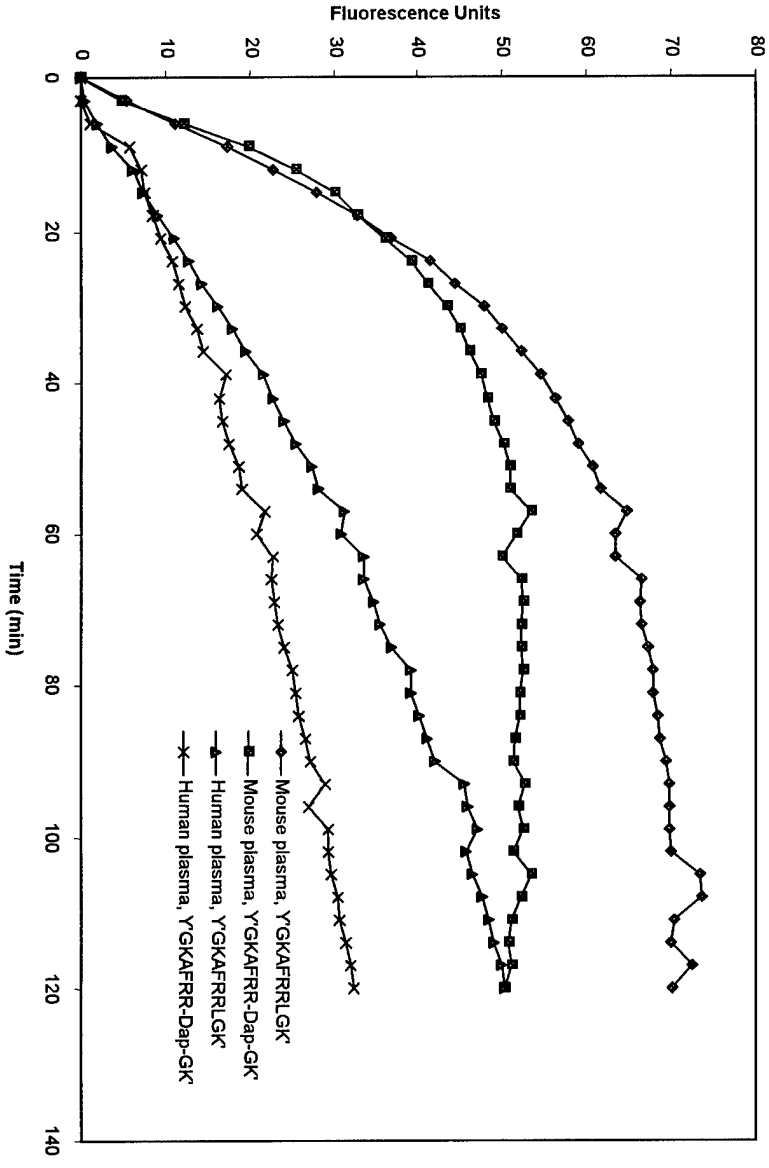
Y'GSKGHFKL-Dap-F-K'

Figure 2

Figure 3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Mouse plasma, Y'GKAFRR-Dap-GK'
Human plasma, Y'GKAFRRLGK'
Human plasma, Y'GKAFRR-Dap-GK'

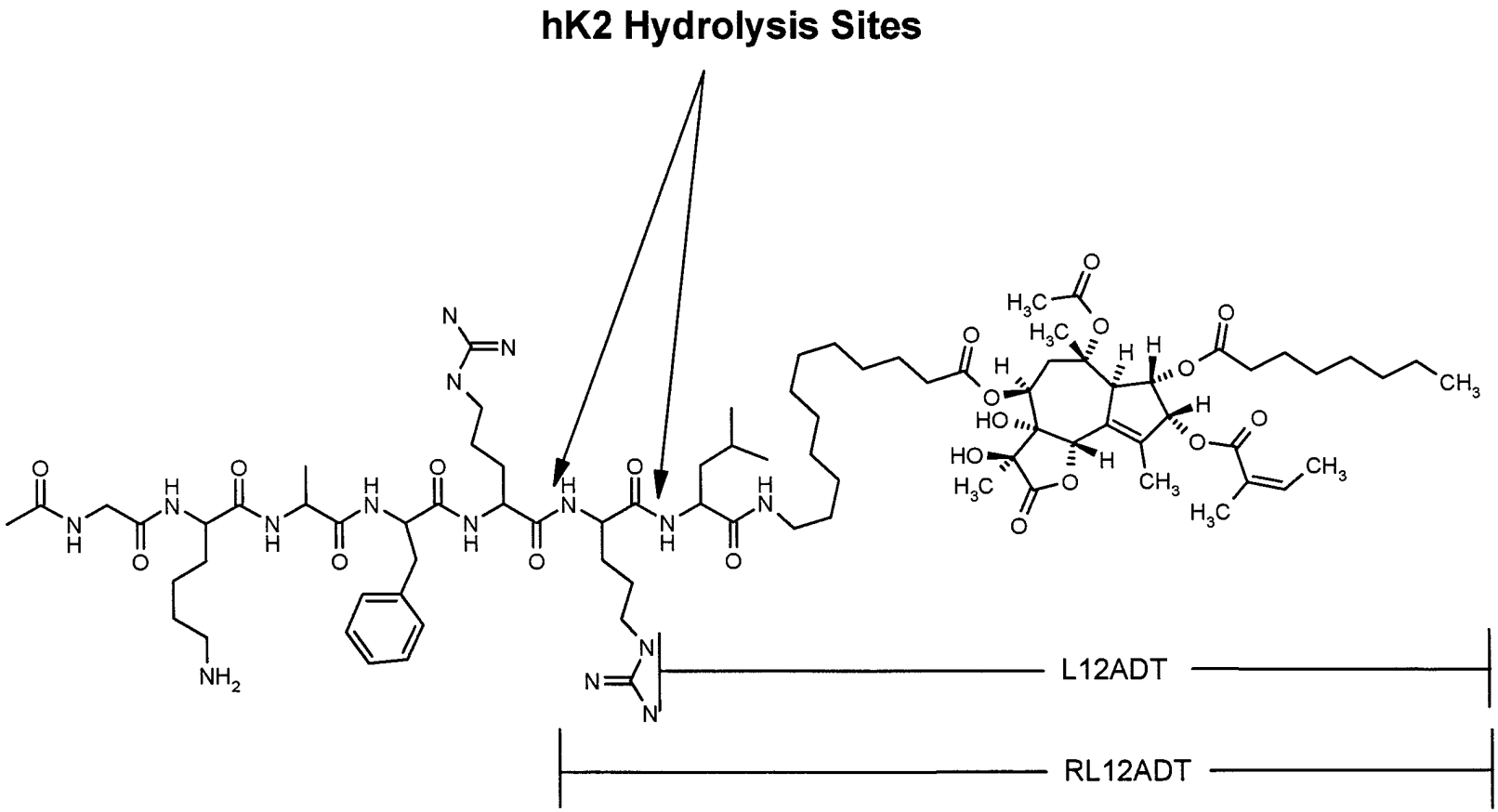
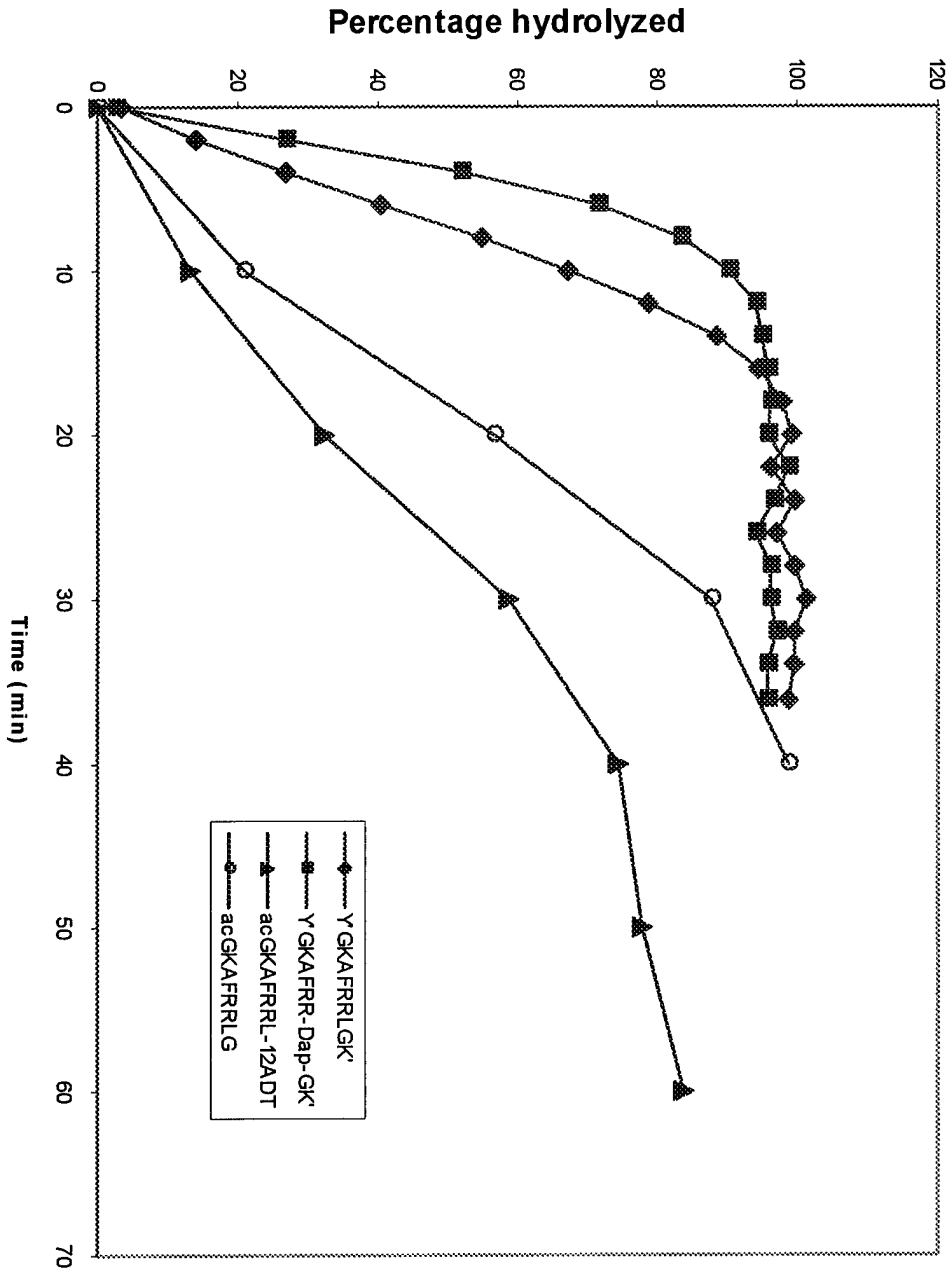


Figure 4

Figure 5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10
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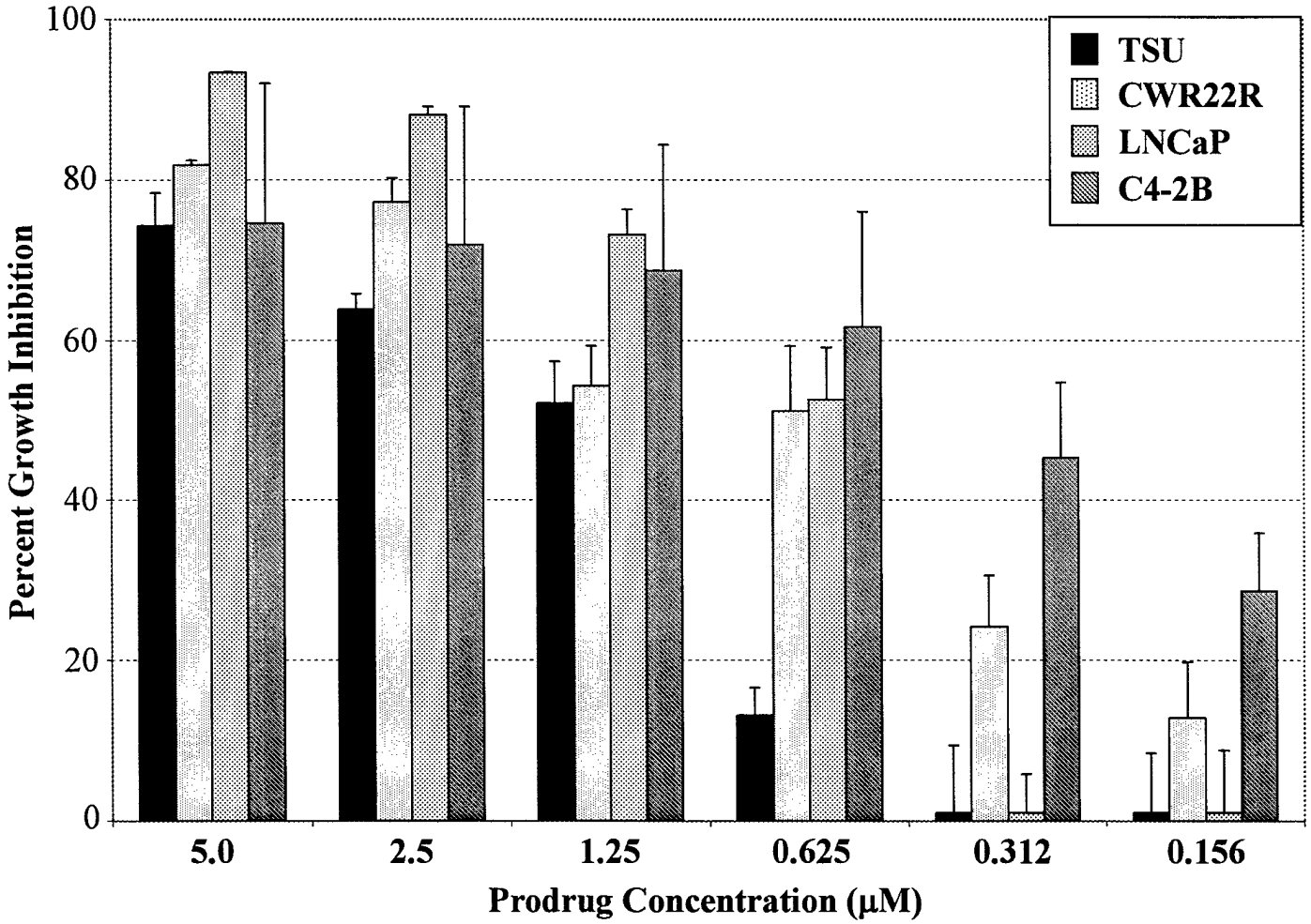
YGKAFRR-Dap-GK'
acGKAFRRL-12ADT
acGKAFRRLG


100
80
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40
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Percent Growth Inhibition
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CWR22R
LNCaP
C4-2B
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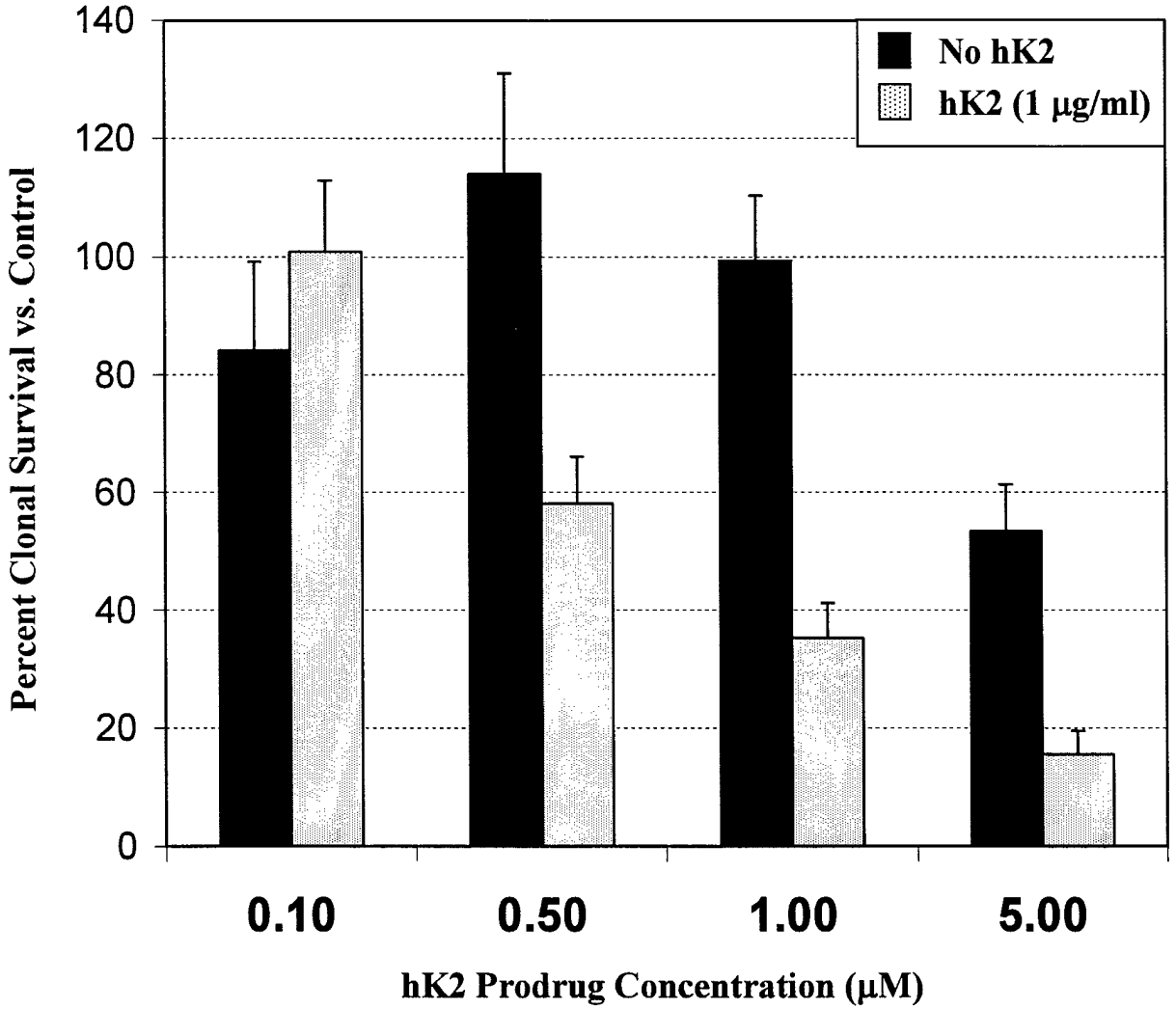
0.156
Prodrug Concentration (μM)

Figure 6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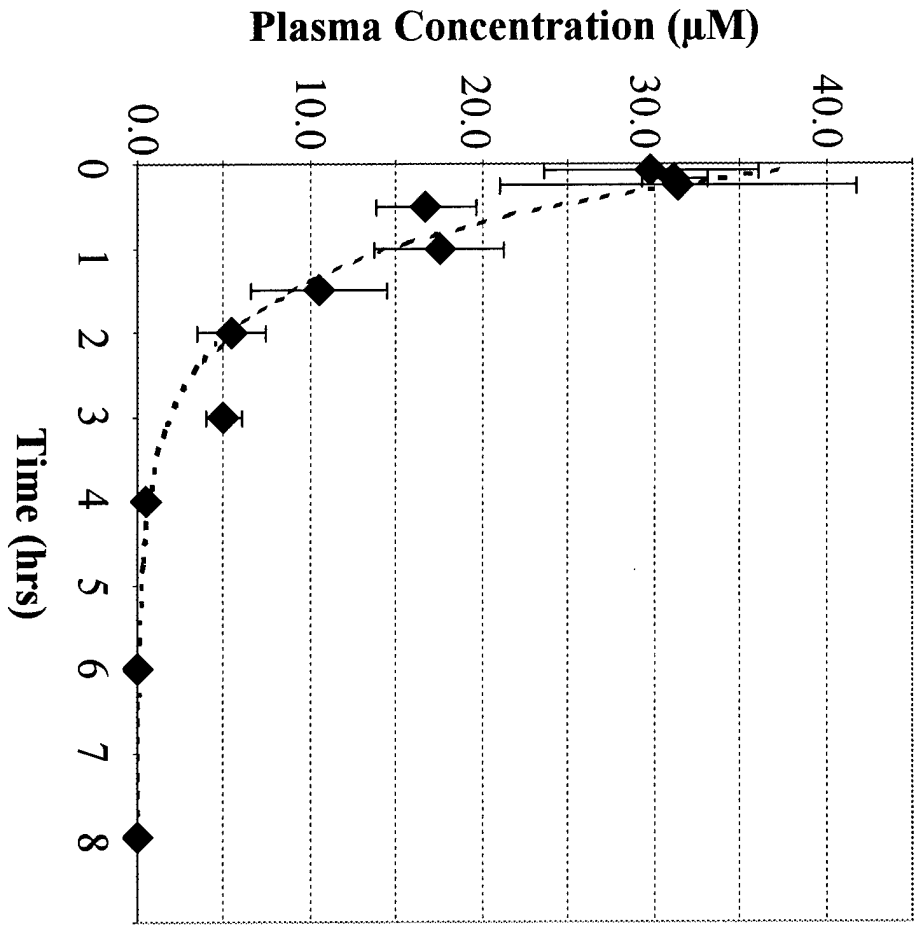
5.00
hK2 Prodrug Concentration (µM)

Figure 7

Figure 8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20.0
30.0
40.0
Time (hrs)
0
1
2
3
4
5
6
7
8

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Microbead display by in vitro compartmentalisation: selection for binding using flow cytometry

Armin Sepp[a,1], Dan S. Tawfik[b,c], Andrew D. Griffiths[a,*]

[a] *MRC Laboratory of Molecular Biology, MRC Centre, Hills Road, Cambridge CB2 2QH, UK*
[b] *Centre for Protein Engineering, MRC Centre, Hills Road, Cambridge CB2 2QH, UK*
[c] *Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot 76 100, Israel*

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Abstract In vitro compartmentalisation in an emulsion was used to physically link proteins to the DNA that encodes them via microbeads. These microbeads can be selected for catalysis, or, as demonstrated here, for binding. Genes encoding a peptide containing an epitope (haemagglutinin) were enriched to near purity from a 10^6-fold excess of genes encoding a different peptide by two rounds of selection using flow cytometry, indicating ~1000-fold enrichment per round. Single beads can be isolated using flow sorting and the single gene on the bead amplified by polymerase chain reaction. Hence, the entire process can be performed completely in vitro.

Key words: In vitro compartmentalization; Microbead; Selection; Binding; Flow cytometry; Directed evolution

1. Introduction

Both natural evolution and directed evolution require a link between genotype and phenotype [1]. This can be achieved in the laboratory by creating a physical genotype–phenotype linkage – either the gene is the active molecule (in the case of nucleic acids [2]) or the gene and the protein it encodes are physically connected.

The first, and most widely adopted technique for selecting protein ligands is phage display, where the protein is displayed on the surface of a filamentous bacteriophage [3]. However, proteins can also be displayed on the surface of other bacteriophage, eukaryotic viruses, bacteria, yeast, and even directly bound to the encoding plasmid [4–7].

Two completely in vitro display technologies have also been developed, which use cell-free translation systems: polysome display [8] and RNA–peptide fusion [9]. They promise much larger repertoire sizes [10,11] and the possibility of selecting proteins with hitherto unattainable properties [12].

We have recently described an entirely different strategy, based on in vitro compartmentalisation (IVC) of reactions in the aqueous droplets of a water-in-oil emulsion [1]. We have previously shown how IVC can be used to select for *catalysis* by selecting DNA-methyltransferases [13] and IVC has also been used for the directed evolution of *Taq* DNA polymerase [14].

Here, we describe a novel IVC strategy based on creating repertoires of microbeads, each displaying a gene and the protein it encodes (Fig. 1). These beads can be selected by the catalytic activity of the displayed protein [29], or, as described here, selected for binding.

2. Materials and methods

2.1. Synthesis of genes

The vector pETFLAG was created by annealing the oligonucleotides FLAG-NB and FLAG-BN (Table 1) and cloning into *Nco*I–*Bam*HI-cut pET23d. The FLAG-haemagglutinin (HA) gene (Fig. 2) was created by polymerase chain reaction (PCR) amplifying pETFLAG with the primers Biotin-pETRev and FLAG-HA. The FLAG-*fol*A gene (Fig. 2A), encoding dihydrofolate reductase (DHFR) with an N-terminal FLAG tag was created by amplifying the *fol*A gene [16] from *Escherichia coli* using primers folA-FW and folA-BW, cloning it into *Hin*dIII–*Xho*I-cut pETFLAG (creating pETFLAG-*fol*A) and amplifying this construct using primers Biotin-pETRev and pETFor.

2.2. Microbead display of peptides

10^9 1.0-μm diameter streptavidin-coated polystyrene beads (Bangs Laboratories) were centrifuged at 2300×*g* for 4 min (as for all washing steps) and then resuspended in 50 μl TNTB (0.1 M Tris 7.5, 0.15 M NaCl, 0.05% Tween-20, 0.5% bovine serum albumin). 8 μg of biotinylated M5 anti-FLAG antibody (Sigma) was added (60 000 antibodies/bead) and incubated for 5 h at 5°C. The beads were washed three times in TNTB before addition of biotinylated FLAG-*fol*A or FLAG-HA DNA at a ratio of two genes/bead, and incubated overnight at 4°C. As ~50% of genes bind (assayed using ^{32}P-labelled DNA), about one gene is bound per bead. The beads were washed twice in TNTB then resuspended in 100 μl of ice-cooled *E. coli* S30 in vitro translation system and emulsified as in [13]. The emulsions were incubated at 30°C for 90 min, spun at 2300×*g* for 10 min and the oil phase removed. 200 μl of TNTB was added and the emulsion broken by extracting three times with 1 ml of hexane. The recovered beads were washed twice in TNTB and resuspended in 0.2 ml of TNTB.

2.3. Labelling of microbeads displaying peptides with an HA epitope

0.5 μl of 25 mU/μl rat anti-HA peroxidase conjugate (3F10, Roche) was added to the recovered beads and incubated for 20 min at 25°C. The beads were washed three times with TNTB then resuspended at 5×10^7 beads per ml in 50 mM Tris–HCl, pH 8.0 containing 0.004% H_2O_2 and 4 μM fluorescein tyramide [17] and sonicated for 1 min. After 5 min at room temperature, the beads were washed three times in TNTB, resuspended in phosphate-buffered saline, sonicated for 1 min and analysed on a FACScan cytometer (Becton Dickinson).

*Corresponding author. Fax: (44)-1223-402140.
E-mail address: griff@mrc-lmb.cam.ac.uk (A.D. Griffiths).

[1] Present address: Domantis Limited, Celltech Building, Granta Park, Great Abington, Cambridge CB1 6GS, UK.

Abbreviations: IVC, in vitro compartmentalisation; TSA, tyramide signal amplification; DHFR, dihydrofolate reductase

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PII: S0014-5793(02)03740-7

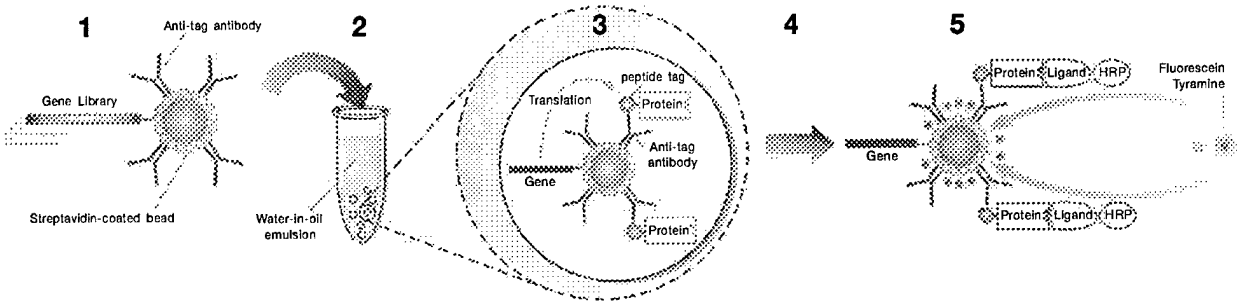

Fig. 1. Creation of microbead display libraries by IVC and selection for binding using flow cytometry. A repertoire of genes encoding protein variants, each with a common N- or C-terminal epitope tag, are linked to streptavidin-coated beads carrying antibodies that bind the epitope tag at, on average, ≤ 1 gene per bead (1). The beads are compartmentalised in a water-in-oil emulsion to give, on average, < 1 bead per compartment (2), and transcribed and translated in vitro in the compartments. Consequently, in each compartment, multiple copies of the translated protein become attached to the gene that encodes it via the bead (3). The emulsion is broken (4), and the microbeads carrying the display library isolated. The beads are incubated with ligand coupled to horseradish peroxidase (HRP), washed to remove unbound ligand and incubated with hydrogen peroxide and fluorescein tyramide (5). Immobilised HRP converts the fluorescein tyramide into a short-lived, free-radical intermediate which reacts with adjacent proteins. Hence, beads displaying proteins that bind ligand become labelled with multiple fluorescein molecules. These beads can then be enriched (together with the genes attached to them) by flow cytometry.

2.4. Sorting of microbeads

Beads were sorted using a MoFlo cell sorter (Cytomation) directly into PCR buffer. DNA was amplified (using Super Taq, HT Biotechnology) with primers Biotin-pETRev and FLAGRev1. 0.5 μl of the first PCR reaction was re-amplified using primers Biotin-pETRevN (which primes inside Biotin-pETRev) and FLAGRev1. The DNA was purified from a 1% agarose gel for a second round of selection. Beads sorted from the second round were amplified using primers Biotin-pETRevN and FLAGRev1 for the first PCR then primers Biotin-pETRevN1 (which primes inside Biotin-pETRevN) and FLAGRev1 for the second. All PCRs were cycled 30 times (94°C, 0.5 min, 60°C, 0.5 min, 72°C, 2.0 min).

Single beads were sorted into individual wells of a 96-well PCR plate into PCR buffer and amplified as above with primers Biotin-pETRev and FLAGRev1 for 37 cycles.

3. Results

3.1. Microbeads displaying multiple copies of a peptide ligand can be created by IVC and identified by flow cytometry

Biotinylated FLAG-HA genes, encoding a 30-residue peptide with a FLAG epitope tag at the N-terminus and an HA epitope at the C-terminus, or FLAG-*fol*A genes, encoding DHFR with an N-terminal FLAG epitope tag (Fig. 2) were attached to 1-μm diameter streptavidin-coated beads coated with anti-HA antibodies at one gene/bead. Microbeads displaying the translated protein were then created by translation in an emulsion and the beads labelled with peroxidase-conjugated anti-HA antibody and fluorescein tyramide signal amplification (TSA) [18] as in Fig. 1.

The beads displaying FLAG-HA peptides could easily be distinguished from the beads displaying FLAG-*fol*A by flow cytometry: the mean fluorescence of beads carrying the FLAG-HA genes was 57 times that of beads with FLAG-*fol*A genes (Fig. 3). A calibration curve constructed by labelling beads coated with biotinylated peroxidase molecules indicated that each bead captured 200–300 FLAG-HA peptides.

Table 1
Oligonucleotides

FLAG-NB	5′-CATGGACTACAAAGATGACGATGATAAAATGC-ATGGCAACGAAGGTACCG-3′
FLAG-BN	5′-GATCCGGTACCTTCGTTGCCATGCATTTTATC-ATCGTCATCTTTGTAGTC-3′
Biotin-pETRev	5′-Biotin-GGTTTTCACCGTCATCACCG-3′
FLAG-HA	5′-AACTCAGCTTCCTTTCGGGCTTTGTTAGGATC-CTCCTCCCGCATAATCCGGCACATCATACGGATA-TCCTCCTCCGGATCCGGTACCTTCGTTGCC-3′
folA-FW	5′-GCGCGAAGCTTCGATCAGTCTGATTGCGGCG-3′
folA-BW	5′-GCGCCTCGAGTTCCGCCGCTCCAGAATCTC-3′
pETFor	5′-GACTCCAACGTCAAAGGGCG-3′
FLAGRev1	5′-AACTCAGCTTCCTTTCGGGC-3′
Biotin-pETRevN	5′-Biotin-AAACGCGCGAGGCAGCTGC-3′
Biotin-pETRevN1	5′-Biotin-GCGAGGCAGCTGCGGTAAAG-3′

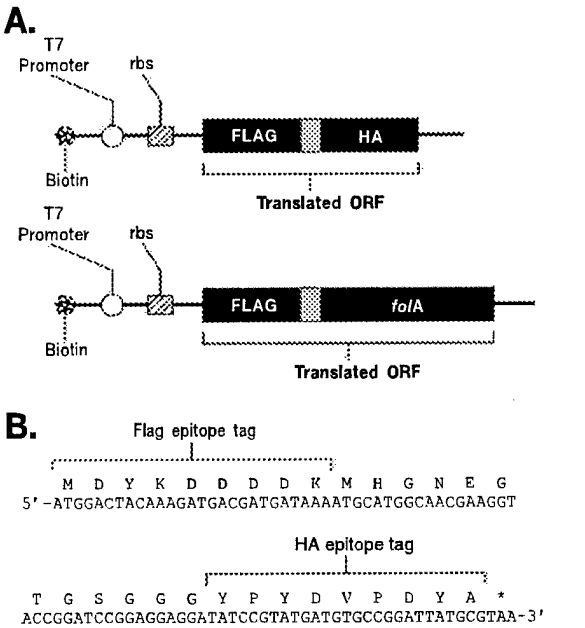

Fig. 2. The selected genes. A: Schematic representation of the gene encoding the 30-residue FLAG-HA peptide with an N-terminal FLAG [15] and a C-terminal HA [28] epitope tag, and the control gene, FLAG-*fol*A, encoding *E. coli* dihydrofolate reductase [16] with an N-terminal FLAG tag. Both genes contain a T7 promoter and ribosome binding site (rbs) and are biotinylated at one end. B: The sequence of the translated open reading frame (Translated ORF) of the FLAG-HA gene.

3.2. A gene encoding a peptide containing an epitope (HA) is enriched to near purity from a 10^6-fold excess of genes encoding a peptide without the epitope after two rounds of selection

Biotinylated FLAG-HA genes and biotinylated FLAG-*fol*A genes (Fig. 2) were mixed in a ratio of $1:10^6$ and attached to beads coated with anti-HA antibodies at one gene/bead, as above. Microbeads displaying the translated protein were then created by translation in an emulsion and the beads labelled with peroxidase-conjugated anti-HA antibody and fluorescein TSA (Fig. 1). 6.6×10^4 beads out of 3.6×10^7 (i.e. $\sim$0.2%) of single, unaggregated beads were sorted using a flow cytometer. The genes on the sorted beads were amplified by PCR using a biotinylated primer, and subjected to a second round of selection as above, except at 0.1 gene/bead. 2.0×10^3 beads out of 2.4×10^5 were collected (i.e. $\sim$1%). After one round of selection a faint band corresponding to the FLAG-HA gene was visible on an agarose gel and after two rounds the FLAG-HA gene had been enriched to near purity (Fig. 4). This indicates an enrichment of about 1000-fold per round. No enrichment was observed when the DNA on the beads was amplified without sorting.

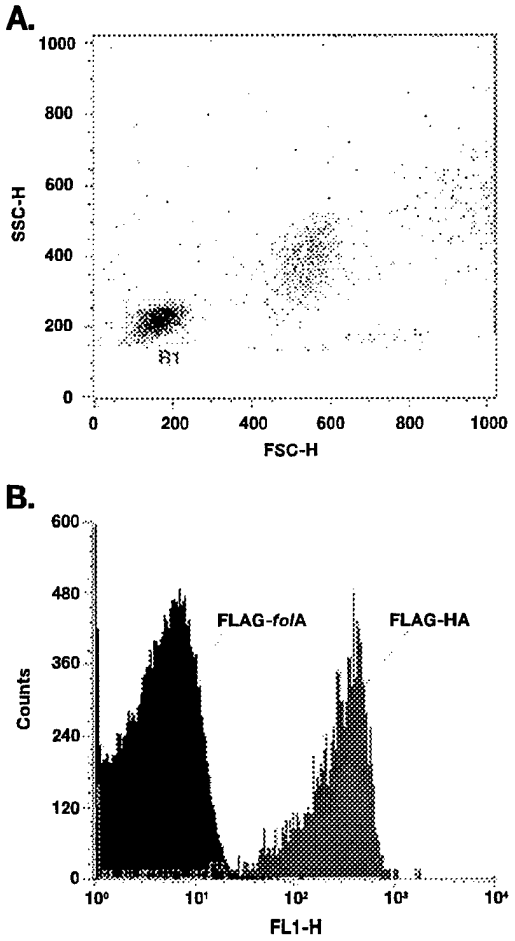

Fig. 3. Flow cytometry of microbead–gene complexes which do, or do not, contain peptides that bind to an anti-HA antibody. A: Forward scatter (FSC-H) and side scatter (SSC-H) of beads analysed by flow cytometry. Single unaggregated beads were gated through R1. B: Fluorescence of beads (gated through R1) coated with either FLAG-*fol*A genes or FLAG-HA genes following translation and capture of peptides onto the beads in an emulsion, incubation with peroxidase-conjugated anti-HA antibody and fluorescein TSA.

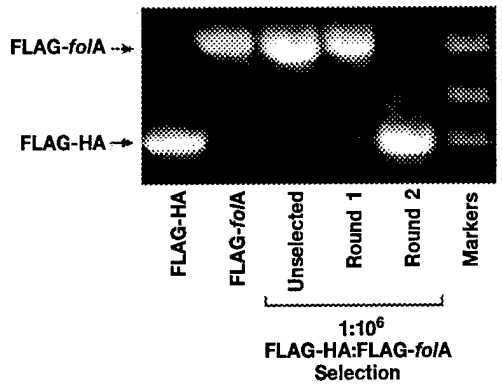

Fig. 4. Selections for genes encoding peptides that bind to an anti-HA antibody from a starting ratio of $1:10^6$ FLAG-HA:FLAG-*fol*A genes. Pure FLAG-HA genes (877 bp), pure FLAG-*fol*A genes (1371 bp), unselected DNA and DNA recovered after round 1 and round 2 of selection were amplified by PCR and analysed on a 1% agarose gel. Markers, ϕX174-*Hae*III digest.

3.3. Individual genes encoding peptides with binding activity can be isolated by single-bead sorting

Beads were coated with a $1:10^3$ mixture of FLAG-HA: FLAG-*fol*A genes at one gene/bead, and selected as above. The most highly fluorescent beads (in the top 0.1%) were sorted by flow cytometry and the genes attached to a pool of 500 sorted beads amplified by PCR (Fig. 5A). After sorting, the band corresponding to the FLAG-HA gene was of

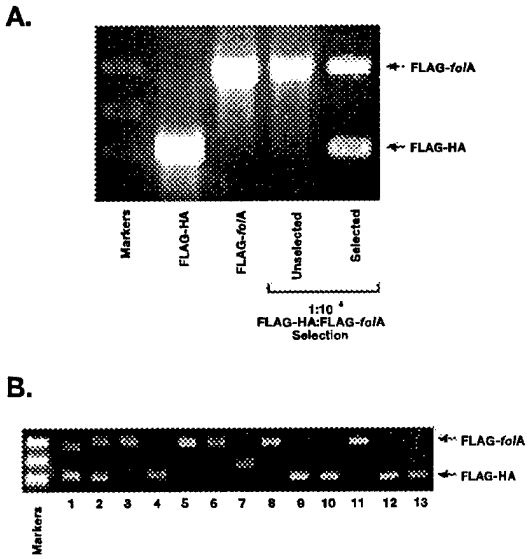

Fig. 5. Direct sorting of individual FLAG-HA genes from a $1:10^3$ mixture of FLAG-HA:FLAG-*fol*A genes by flow cytometry of beads. A: Pure FLAG-HA genes (903 bp), pure FLAG-*fol*A genes (1397 bp), unselected DNA and DNA recovered from 500 sorted beads (pooled together) after one round of selection were amplified by PCR and analysed on a 1% agarose gel. B: DNA amplified by PCR from *single* positive beads sorted into the wells of a 96-well PCR plate, 13 of which are shown analysed on a 1% agarose gel. Markers, ϕX174-*Hae*III digest.

similar intensity to the band corresponding to the FLAG-*fol*A genes indicating an enrichment of ~500-fold. No enrichment was observed when the beads were not sorted. In addition, *single*, highly fluorescent beads (in the top 0.1%) were sorted into the wells of a 96-well PCR plate. DNA was successfully amplified by PCR from 22 of the 96 wells and 13 of these are shown in Fig. 5B. Hence, ~23% of the input genes were recovered intact. Of the wells that amplified, nine gave single bands of the size expected for FLAG-HA, and 11 gave single bands of the size expected for FLAG-*fol*A. This indicates that the FLAG-HA genes were enriched ~500-fold and is consistent with the enrichments observed above (Figs. 4 and 5A).

4. Discussion

We demonstrate here how IVC provides an efficient way of physically linking proteins to the genes that encode them and selecting them for binding. IVC has also been used for the selection of gene–protein complexes created by translation of biotinylated genes encoding peptides fused to streptavidin [19]. However, the enrichment observed in a model selection was very low (10-fold; v. >500-fold observed here) and the efficiency of formation of the protein–DNA complexes was only ~1%.

Proteins can of course be physically linked to genes that encode them by a variety of techniques. These 'display technologies' have proven highly successful in the selection of binding proteins [10,12,20–25]. Nevertheless, microbead display libraries made by IVC have a number of attractive features.

First, they are formed completely in vitro; hence no transformation or cloning is required (unlike systems with an in vivo step such as phage display [20,21]).

Second, the gene is DNA, which is more stable than the RNA used in other completely in vitro systems [10,12,25]. Indeed, 23% of the input genes survived intact at the end of the selection.

Third, the efficiency of assembly is high. Nearly all the beads coated with FLAG-HA, translated in an emulsion and labelled using peroxidase-conjugated anti-HA antibody and fluorescein TSA, were highly fluorescent (Fig. 3B). Furthermore, in the selection of a $1:10^6$ FLAG-HA:FLAG-*fol*A gene mixture, most of the beads (at least one in eight) carrying FLAG-HA genes must have become labelled with fluorescein and sorted (as 23% of genes survived, only 8×10^6 of the 3.6×10^7 beads sorted carried a gene).

Fourth, multiple copies of each protein can be displayed (as opposed to single copies in ribosome display or mRNA–peptide fusion) providing a potential advantage in the selection of low affinity ligands. In this case 200–300 peptides were displayed per bead.

Fifth, with other display technologies the peptides to be selected are fused to proteins for display on phage or cells, attached to ribosomes or fused to mRNA and this can lead to the selection of peptides with poor solubility [12]. With microbead display the expressed peptides are only fused to a short epitope tag and peptides that are too insoluble to be captured by the beads will not to be selected.

Finally, the microbead display libraries can be selected by flow cytometry, which has a variety of practical advantages for the selection of ligand binding [26]. Ligand binding equilibria and dissociation kinetics can be determined and clones selected accordingly. Indeed, flow cytometry has been used to select an extremely high affinity anti-fluorescein single-chain Fv antibody ($K_d = 48$ fM) from libraries displayed on yeast [27].

Throughput is relatively high (up to 100 000 s^{-1}; http://www.cytomation.com), but flow cytometry does impose an upper limit of $\sim10^9$ on the size of libraries that can be selected. The small size of the compartments (~5 fl) means that very large gene libraries could potentially be selected: a 100 μl reaction mix dispersed in 0.5 ml oil forms $\sim2\times10^{10}$ aqueous compartments. However, to do so it is probably necessary to select the microbead display libraries by affinity purification, as with other display technologies.

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